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Vol. 8. No. 4. DECEMBER 1953

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BRITISH JOURNAL OF PHARMACOLOGY AND CHEMOTHERAPY

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LONDON
BRITISH MEDICAL ASSOCIATION
TAVISTOCK SQUARE, W.C.1

YEARLY SUBSCRIPTION (4 NUMBERS) £4 4s. U.S.A. $13.50 SINGLE NUMBERS £1 5s.

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Brit. J. Pharmacol. (1953), **8**, 371.

PLASMA IRON LEVELS AND URINARY IRON EXCRETION AFTER THE INTRAVENOUS ADMINISTRATION OF DIFFERENT IRON PREPARATIONS*

BY

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(RECEIVED NOVEMBER 8, 1952)

The present study of plasma iron levels and urinary iron excretion after intravenous administration of different iron preparations was carried out to elucidate the fate of injected iron and obtain a better understanding of the mechanisms of toxicity of the different preparations. The doses used were near the maximum tolerated doses, and the compounds included both well-known and new preparations (Nissim, 1949, 1953).

METHODS

Serum and plasma iron were determined by the ortho-phenanthroline method (Laurell, 1947), modified in some details. Thus, the amount of HCl in this method (3 ml. 6N HCl to 6 ml. serum) converted only 17.5% of saccharated iron oxide into ferric chloride, whereas complete breakdown required 9 ml. HCl to 6 ml. of solution. At first estimations were carried out on blood serum, but inaccurate results were obtained with ferric glucosate, since the particles clumped and adhered to coagulating proteins during clotting. All subsequent iron estimations were made on plasma.

Ortho-phenanthroline did not dissolve in 10% absolute alcohol to give a 2% solution, and a 1% solution in 30% absolute alcohol was used instead. Analar sodium sulphite was used instead of sodium hydrosulphite : this made the solution alkaline, and 0.5N-HCl was added to get rid of the yellow paranitrophenol colour before the addition of ortho-phenanthroline. The high plasma iron values also necessitated the addition of more ortho-phenanthroline and sodium sulphite to ensure the estimation of all the iron. The amount of plasma was reduced from 6 to 0.5 ml. in order to use the steep part of the absorptiometer curve.

Effect of Haemolysis on Serum Iron Values.—High plasma iron increased the tendency of the blood to haemolyse *in vitro*. The effect of haemolysis on serum iron values was therefore investigated.

Rabbit blood (7.5 ml.) was partly haemolysed with 2.5 ml. hypotonic saline to produce a deep pink colour. Serum iron was 0.28 mg. per 100 ml. after allowing for dilution. This is not much higher than serum iron values in normal rabbits (0.10–0.25 mg. per 100 ml.).

Red cells from 4 ml. of rabbit blood were washed repeatedly with normal saline, haemolysed completely with saponin, and treated with 12 ml. conc. HCl. The iron contributed by the haemolysed cells was 0.62 mg. per 100 ml. blood, which agrees with the values of non-haemoglobin iron present in red cells (Shortland and Wall, 1936). Even conc. HCl, therefore, failed to split the haemoglobin molecule. The accidental haemolysis of even one-tenth of the red cells would introduce an error of 0.06 mg. per 100 ml., which is negligible in experiments dealing with levels of 1 to over 100 mg. per 100 ml.

Estimation of Iron in Urine.—Iron estimations were carried out as for plasma on 0.5 ml. of urine from two normal rabbits. The iron content was negligible ; it could not be estimated accurately with this method because of interference from pigments. Urinary iron excretion after iron injections was, however, high enough for comparative estimations to be made with the same simple method which was used for plasma.

Modified Phenanthroline Method used in the Present Experiments.—Blood (3–4 ml.) is mixed in a graduated centrifuge tube with 0.1 ml. of 1% heparin, and centrifuged for 15 minutes. The volume of plasma is noted, and 0.5 ml. is transferrred to a round-bottomed 50-ml. centrifuge tube ; 1.5 ml. conc. HCl is added, followed in 10 minutes by 3 ml. of 20% redistilled trichloracetic acid. The mixture is centrifuged until the supernatant fluid is clear. The latter is then poured into a 12.5-ml. volumetric flask, a drop of 1% paranitrophenol is added as indicator, followed by conc. ammonia, drop by drop, until a yellow colour is just obtained. The mixture is acidified with N/2 HCl until the colour disappears, buffered with 0.2 ml. of 1N sodium acetate, and reacidified with N/2 HCl until the colour disappears again. Fifteen drops of 1% ortho-phenanthroline are added, followed by 150 mg. of sodium sulphite. When the latter has dissolved, the solution is reacidified with N/2 HCl until all yellow colour has disappeared. Distilled water is now added to the mark, and, after mixing, the intensity of

*This work formed part of an M.D. thesis approved by the University of London.

TABLE I

PLASMA IRON LEVELS AND URINARY IRON EXCRETION AFTER THE INTRAVENOUS INJECTION OF DIFFERENT IRON PREPARATIONS INTO RABBITS

Expt.	Compound	Weight of Rabbit in kg.	Dose mg. Fe/kg.	Fe Injected in mg.	Plasma Iron (Hours after Injection) 0	½	3	6	24	Quantity Excreted in 24 hrs., mg. Fe	% Excreted
1 a	Saccharated iron oxide (G_4) ..	2·9	45	130	125	95	70	45	5·4	—	—
b	Iron and ammonium citrate ..	2·6	45	117	110	41·5	14	7	0·5	—	—
2 a	Saccharated iron oxide (G_4) ..	2·1	45	94	120	95	41	24	1	3·0	3·2
b	Iron and ammonium citrate ..	2·7	45	122	112	32	10	5	1	46·5	38·1
3 a	Saccharated iron oxide (G_4) ..	2·05	22·5	46	62	42	28	10	—	1·15	2·5
b	Iron and ammonium citrate ..	2·4	22·5	54	45	17	7	2	—	8·7	16·1
4 a	Saccharated iron oxide (G_4) ..	2·1	180	380	340	144	56	44	—	8·25	2·2
b	,, ,, ,, ,, ..	2·3	360	828	500	375	175	—	—	16·5	2·0
5 a	Saccharated iron oxide (Sample B)	3·0	22·5	66	49	31	11·5	5	—	2	3·0
b	,, ,, ,, ,, ..	2·3	22·5	50	46	34	13	5·5	—	1·9	3·8
6 a	Ferrivenin	3·0	22·5	66	46	32·2	12·3	5·3	—	2·2	3·0
b	,, ...	2·3	22·5	50	39	29	16	4	—	1·63	3·3
7 a	Ferric glucosate	2·0	22·5	45	65	50	21·5	7·5	—	1·2	2·7
b	,, ,, ...	3·0	45	135	100	67	—	—	—	—	—
c	,, ,, ...	2·2	90	200	141	63	—	—	—	—	—
8 a	Ferric hydroxide ferrous ascorbate	2·35	22·5	51	22	8	4	2	—	14·0	27·4
b	Ferrous chloride ascorbate ..	3·15	22·5	71	41	27	—	—	—	—	—
9 a	Ferrous ascorbate	3·6	22·5	82	50	40	20	—	—	1·0	1·5
b	Neutral ferrous ascorbate ..	2·5	22·5	56	39	30	17	13	—	4·0	7·1
c	Alkaline ferrous ascorbate ..	2·5	22·5	56	36	32	19	17	—	4·1	7·3
10 a	Colloidal ferric hydroxide ..	2·05	22·5	46	11	9	1·25	0·9	—	0·125	0·27
b	Ferric chloride caramelate ..	2·8	22·5	62·4	46	25	15	8	—	11·67	18·7
11 a	Ferrous chloride pyruvate ..	2·5	22·5	56	42	23	5	2·5	—	0·16	0·29
b	Ferric chloride lactate ..	2·4	22·5	54	65	61	41	12	—	0·8	1·5
12 a	Ferric tartrate	2·58	22·5	58	45	32	17·5	10·5	—	2·04	3·5
b	Ferronascine	2·75	22·5	62	50	38	25	16	—	2·15	3·5

The concentration of Fe in the injected solutions varied between 0·5 and 2%.

the colour is estimated by the photoelectric absorptiometer, using a filter with maximum transmission of light at wavelength 490 mμ. The reading is made against a control of distilled water treated similarly.

RESULTS

Plasma or serum iron levels and urinary iron excretion with different iron preparations are shown in Table I. The following points call for comment.

Saccharated Iron Oxide (G_4) and Iron and Ammonium Citrate.—In each experiment two rabbits were used, one injected intravenously with saccharated iron oxide, the other with iron and ammonium citrate (both 45 mg. Fe/kg.). In Experiment 1 estimations were carried out on serum, in Experiment 2 on plasma (Table I).

A wide difference is noted in the disappearance of iron from the blood with the two preparations. Indeed, if the first sample of blood after iron and ammonium citrate had not been collected speedily, the initial high figures would have been missed. If plasma volume in rabbits is taken as 3.5–4.5% of the body weight, a dose of 45 mg./kg. would give a plasma content of 100–130 mg./100 ml., provided the whole dose remains in solution in the blood. The first figures for both preparations were within these theoretical values. With iron and ammonium citrate, however, the rate of disappearance of iron was so rapid that the figures for the first estimations (110 and 112 mg.) were very likely less than the true theoretical values. The rabbits remained alive and well after the injection. Injection of half the dose of iron gave qualitatively similar results.

Plasma Iron Levels and Urinary Iron Excretion after Intravenous Injection of Different Doses and Samples of Saccharated Iron Oxide.—Two rabbits were injected intravenously with 180 and 360 mg. Fe/kg. of saccharated iron oxide (G_4) respectively (Table I, Experiment 4). The first rabbit died accidentally eight hours after injection, as a result of a haemopericardium from a heart puncture performed for collecting the last sample of blood; the second rabbit died, shortly after collection of the three-hour sample, with pulmonary and systemic haemorrhages.

Relative to the dose injected, the immediate plasma iron levels were not as high as they should have been on theoretical calculations. By comparison with the smaller doses of 22.5 and 45 mg. Fe/kg. previously given, the first levels should have been about 480 and 960 mg. %. The actual figures of 340 and 500 obtained lead to the conclusion that from one-quarter to one-half of the injected iron was precipitated at once at the dose levels of 180 and 360 mg./kg. respectively, the proportion of immediate precipitation increasing rapidly with the dose. The rate of fall of plasma

iron after these doses is also much more rapid than with the smaller doses of 22.5 and 45 mg. Fe/kg. The plasma iron level of 500 mg. % is the highest ever recorded.

The amount of iron excreted in the urine following increasingly larger doses of saccharated iron oxide shows a steady and appreciable rise, but the actual percentage of excretion shows some decline. This is perhaps caused by iron precipitates blocking capillaries in the kidney. With truly diffusible preparations such as iron and ammonium citrate, a rise in the total amount, as well as in the percentage of iron excreted, has been obtained.

In Experiment 5, Table I, two rabbits were given intravenously a dose of 22.5 mg. Fe/kg. of sample B of saccharated iron oxide (Nissim and Robson, 1949), and another two were given the same dose of Benger's saccharated iron oxide (" Ferrivenin "). Both these samples (precipitation point at *p*H 5.7 and 5.8 respectively) gave rise to a lower plasma iron than that after sample G_4 (p. pt. at *p*H 3.7). For a quantitative comparison, the average of the two half-hour values obtained with each sample of saccharated iron oxide was studied. The half-hour values are more likely to give accurate results, since by that time the rate of fall of the plasma levels has slowed down considerably, and speed in collecting the blood samples is not so vital as with the first set of values. The half-hour averages are:

$$\text{Saccharated iron oxide } (G_4) = \frac{40 + 42}{2} = 41$$

$$\text{Saccharated iron oxide (B)} = \frac{34 + 31}{2} = 32.5$$

$$\text{Ferrivenin} = \frac{32 + 29}{2} = 30.5$$

The differences appear to be significant. Neither the histological findings nor the urinary excretion suggest that this more rapid fall is due to greater diffusibility. For this reason, and in view of the greater toxicity of sample B and ferrivenin, these lower figures are indicative of greater precipitation. The lower initial values are compatible with some degree of abrupt precipitation at this dose level, comparable to that seen with sample G_4 with doses of 180 and 360 mg. Fe/kg.

Colloidal Ferric Hydroxide.—One rabbit injected with 45 mg. Fe/kg. intravenously of 2% Fe solution of colloidal ferric hydroxide (" Colliron "-Evans) died in two minutes. Blood withdrawn by heart puncture gave a serum iron of 20 mg. %, only about a sixth of the theoretical value. Death was no doubt due to the massive precipitation known to occur with colloidal ferric hydroxide. Even the low figure of 20 mg. % could not entirely have been due to iron remaining in solution, but must have included fine floccules floating in the blood.

Ferric Glucosate.—A rabbit injected with 45 mg. Fe/kg. intravenously of 2% Fe solution of ferric glucosate died in two hours with haemoptysis. Post-mortem examination showed massive haemorrhages confined to the lungs. A dose of 22.5 mg. Fe/kg. was not fatal (Table I).

Two other rabbits were injected intravenously with 45 and 90 mg. Fe/kg. of ferric glucosate respectively. Both died before the three-hour samples could be collected (Table I), their survival times being 2½ and 1½ hours respectively. The theoretical values for the first samples corresponding to these doses are 120 and 240 mg. %, but from the actual figures obtained, viz., 100 and 141 mg. %, it appears that abrupt precipitation begins to occur at lower plasma levels than with saccharated iron oxide. The half-hour plasma levels show that the rate of fall of plasma iron concentration is also correspondingly more rapid.

" Ferric Hydroxide Ferrous Ascorbate."—Urinary elimination of " ferric hydroxide ferrous ascorbate " was remarkably high, and, because of the dark colour of the preparation, urine passed within the first half-hour was almost black. Most of the iron in the urine was excreted in that period as later samples were near normal in colour. The rabbit remained alive and well.

" Ferrous Chloride Ascorbate."—This rabbit died 40 minutes after the injection. Its bladder contained 6 ml. urine, and iron estimation gave a urinary excretion not higher than 0.63%. The difference between this preparation and " ferric hydroxide ferrous ascorbate " was unexpected. Although the rabbit injected with " ferrous chloride ascorbate " died within 40 minutes, its urinary excretion in 24 hours is not likely to have exceeded 2%. That this low urinary iron excretion after " ferrous chloride ascorbate " was not due to the early collapse and rapid death of the animal is shown by the fact that rabbits which were injected with large doses of " ferric hydroxide ferrous ascorbate " and which died as early as half an hour after the injection showed a urinary iron excretion as high as 20%. The two iron preparations are thus exactly opposite in this respect. Plasma iron levels of " ferrous chloride ascorbate " were higher and more maintained than those of " ferric hydroxide ferrous ascorbate." This fact is of significance, and it may be

safely concluded that the greater toxicity and lower urinary excretion of this preparation is not due to massive precipitation as with colloidal ferric hydroxide. Other more important factors are at work.

Neutral and Alkaline Ferrous Ascorbate.—One rabbit injected with freshly prepared ferrous ascorbate (Maurer and Schiedt, 1936) died in 12 hours. Post-mortem examination showed lung haemorrhages, though not as marked as with "ferrous chloride ascorbate," and considerable oedema with areas of collapse.

Urinary iron excretion did not exceed 1.5%, in contrast to the 27.4% of "ferric hydroxide ferrous ascorbate." This was not merely a fortuitous result. While carrying out toxicity studies on mice, the excretion of "ferric hydroxide ferrous ascorbate" was found to be so rapid that the urine turned black at the end of the intravenous lethal doses of 90 mg. Fe/kg., provided these were given slowly in 5–10 minutes. This finding was made use of as a rough test for urinary excretion of iron-ascorbic acid preparations. The urine of mice injected with fresh neutral ferrous ascorbate remained clear amber in colour, while samples of ferrous ascorbate kept in solution for 2–3 days showed increased urinary elimination. This was also borne out by the following experiments on rabbits.

Two rabbits were injected with two samples of ferrous ascorbate which had been allowed to stand for a few days, one in neutral solution, the other at *p*H 11. The latter provided a control for "ferric hydroxide ferrous ascorbate" also of *p*H 11. The iron content of filtered samples of both neutral and alkaline ferrous ascorbate was estimated before injection, as some of the iron precipitated on standing. The first rabbit died on the second day with some pleural effusion, solid oedematous lungs, but no macroscopic haemorrhages. The second rabbit died on the third day with similar changes.

The increased urinary elimination of these samples of ferrous ascorbate stopped short of that shown by "ferric hydroxide ferrous ascorbate," however prolonged the period of keeping was. There is some evidence, nevertheless, that the excretion of the latter may be approached by samples of ferrous ascorbate with greater proportions of ascorbic acid.

"Ferric Chloride Lactate."—This rabbit died in six hours with copious outpouring of frothy blood-stained fluid from the mouth and nostrils. Post-mortem revealed haemorrhagic areas in the lungs with considerable oedema and some pleural effusion.

"Ferronascine" (Roche).—This rabbit died on the third day, with the same triad of patchy haemorrhage, oedema, and collapse in both lungs.

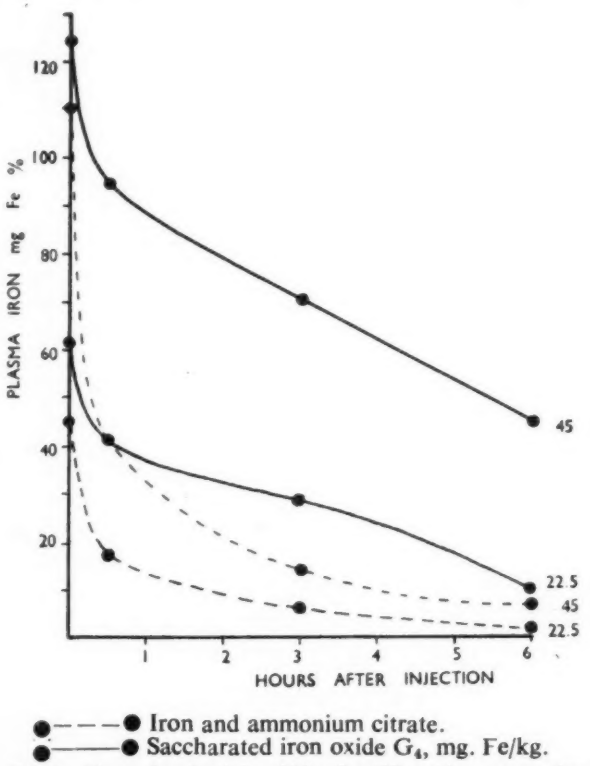

FIG. 1.—Plasma iron levels in rabbits after different iron preparations given intravenously in doses of 22.5 and 45 mg. Fe/kg.

Plasma Iron Levels in Rabbits with Ligated Renal Vessels

When the plasma iron curves of saccharated iron oxide and iron and ammonium citrate (Fig. 1) are compared, the question arises whether urinary excretion can account for the whole difference in their rates of decline. If 62 mg. Fe % is taken as the theoretical plasma level immediately after the injection of 22.5 mg. Fe/kg. of both preparations (Table I, Experiment 3), it may be calculated that the difference between their levels at the half-hour estimation is equal to 40.3% of the total dose of iron and ammonium citrate. Even if all the iron appearing in the urine were eliminated in the first half-hour, the difference between their urinary excretion could only account for 13.6%, leaving 26.7% to be accounted for. In order to obtain a definite answer, plasma iron levels were studied after ligation of renal vessels.

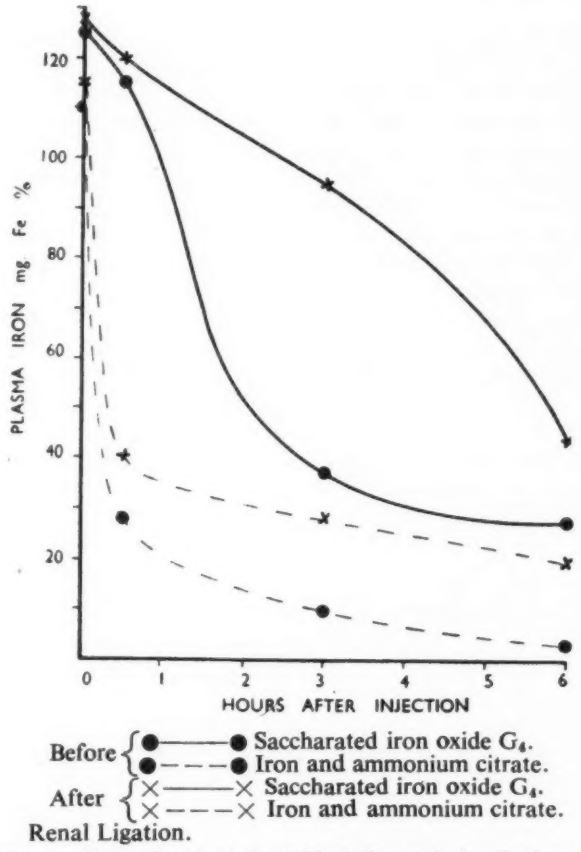

FIG. 2.—Plasma iron levels in rabbits before and after ligation of renal vessels, following saccharated iron oxide (G_4) and iron and ammonium citrate given intravenously in doses of 45 mg. Fe/kg.

Two rabbits were injected intravenously with 45 mg. Fe/kg. of saccharated iron oxide and iron and ammonium citrate respectively, shortly after their renal arteries and veins had been ligated on both sides; the plasma iron levels are shown in Table II, Experiment 1. Both animals were killed at the end of the experiment. Their renal vessels were found to be well ligated and their bladders empty. As expected, the fall of plasma iron was more rapid after iron and ammonium citrate. An unexpected feature was the slowing in the fall of plasma iron in both rabbits.

Two pairs of rabbits were injected intravenously with 45 and 22.5 mg. Fe/kg. of saccharated iron oxide and iron and ammonium citrate respectively, and their plasma iron levels and urinary iron excretion estimated. A fortnight later bilateral ligation of the renal vessels was performed, and shortly after recovery of the animals from anaesthesia they were injected with the same dose of their respective preparations. Plasma iron levels were estimated as before, the animals were killed at 24 hours, and faulty renal ligation was excluded. The whole of the alimentary tract was removed after ligating both ends, and rinsed free from blood. Its contents were added to the faeces passed in the 24 hours by the corresponding rabbit, and the mixture dried and powdered. An aliquot portion was ashed, and iron estimated by the phenanthroline method (Table II).

The considerable slowing in the fall of plasma iron after the ligation operation is more than can be accounted for by retention of iron previously excreted in the urine. Iron uptake by the kidneys was excluded by histological examination, as well as by a control experiment with saccharated iron oxide, in which the ureters were ligated instead of the renal vessels; the slowing was again evident. This slowing may be presumed, therefore, to be due to depression of the activities of the reticulo-endothelial system, and the metabolic processes in general, as a result of uraemia, the anaesthetic (ether), or both.

The iron present in the faeces plus alimentary tract contents was almost the same for both rabbits in both experiments, and any excretion of

TABLE II

PLASMA IRON LEVELS AND IRON EXCRETION IN RABBITS AFTER INTRAVENOUS INJECTION OF SACCHARATED IRON OXIDE (S.I.O.) AND IRON AND AMMONIUM CITRATE (I.A.C.) BEFORE AND AFTER LIGATION OF RENAL VESSELS

Experiment	Compound	Weight of Rabbit in kg.	Fe Injected in mg.	Plasma Iron (Hours after Injection)					Quantity Excreted in Urine in 24 hrs., mg. Fe	Quantity Excreted in Faeces in 24 hrs., mg. Fe
				0	½	3	6	24		
1. After renal ligation ..	S.I.O.	3·3	74	125	110	100	95	5	—	—
	I.A.C.	3·5	79	120	72	45	20	—	—	—
2. Before renal ligation ..	S.I.O.	2·4	108	125	115	37	27·5	1·5	4·2	—
	I.A.C.	2·1	94	110	28	9·5	3	0·5	36	—
After renal ligation ..	S.I.O.	2·4	108	128	120	95	44	—	—	29·5
	I.A.C.	2·1	94	115	40	28	19	—	—	31·2
3. Before renal ligation ..	S.I.O.	2·2	50	64	40	25	8	—	1·9	—
	I.A.C.	2·05	46	39	14	6	2	—	7·6	—
After renal ligation ..	S.I.O.	2·2	50	66	61	31·5	8·7	—	—	25·8
	I.A.C.	2·05	46	44·5	23·5	9	4	—	—	25·6

In Experiments 1 and 2 a dose of 45 mg. Fe/kg., and in Experiment 3 22.5 mg. Fe/kg. was used. The concentration of the solutions injected was 2 g. Fe/100 ml.

iron and ammonium citrate via the bowel could not adequately explain the rapid fall of plasma iron with this preparation. If excretion by this route were to explain the difference in plasma iron at the half-hour estimations, the figure for the iron and ammonium citrate rabbit would have to be 58.8 mg. in the first experiment and 25.8 mg. in the second.

The total iron of the faeces plus alimentary tract contents in all four rabbits was high. As no data were available on faecal iron in rabbits, control estimations were carried out on two normal rabbits of the same weight, their 24-hour faeces and alimentary tract contents collected, and the iron estimated as before. One rabbit gave an iron content of 28.5, the other 32.6 mg. It was realized that the iron in the diet of the rabbits (rabbit diet 18) was high, and in fact proved to be 13 mg. % of its dry weight. Faecal iron in normal rabbits on this diet was 57 mg. % even without ashing. For exact determination of percentage iron excretion via the bowels, rabbits should be kept on iron-free diet, or radio-iron used. The above experiments show, however, that iron excretion via the alimentary tract must be very small even with the massive doses of intravenous iron preparations used here.

Diffusion of Different Iron Preparations into Tissue Fluids

The easy passage of iron through the kidneys with preparations showing a high urinary iron excretion suggested that diffusion of the injected iron into tissue fluids might also take place. The diffusibility of some iron preparations was therefore studied, using the live peritoneum as a dialysing membrane. Mice were injected with 2.0 ml. physiological saline intraperitoneally, and then, immediately afterwards, with the iron preparation intravenously. The latter was given over a period of ten minutes; in this way larger doses of the more toxic preparations could be administered. At the end of the injection the mice were killed, the peritoneal cavity was opened, and 1.0 ml. of fluid withdrawn. Great care was taken to avoid the contamination of this fluid with blood. The iron content was estimated with the phenanthroline method (Table III).

TABLE III

DIFFUSION INTO THE PERITONEAL CAVITY OF DIFFERENT IRON PREPARATIONS INJECTED INTRAVENOUSLY

Iron Preparation	Dose Injected (mg. Fe/kg.)	Iron Concentration in Peritoneal Fluid (mg. Fe %)
1. Saccharated iron oxide (G_4) ..	180	0·25, 0·18
2. Iron and ammonium citrate ..	180	12·5, 7·5
3. Ferric glucosate	180	0·35
1. Saccharated iron oxide (G_4) ..	90	0·2, 0·15
2. Iron and ammonium citrate ..	90	4·3, 6·5
3. Ferric hydroxide ferrous ascorbate	90	7·2, 7·3, 8·6, 4·5
4. Ferric chloride caramelate ..	90	4·0, 5·5, 4·5
5. ,, tartrate	90	2·0, 2·0
6. Ferronascine	90	2·0, 1·5
7. Ferric chloride lactate ..	90	0·4, 0·3
8. ,, glucosate	90	0·24, 0·16

The three most diffusible preparations are those that showed the highest renal elimination, headed by "ferric hydroxide ferrous ascorbate." The diffusibility of saccharated iron oxide, ferric glucosate, and "ferric chloride lactate" is negligible. Ferric tartrate and Ferronascine occupy an intermediate position, which is not matched, however, by their relative rates of renal elimination.

Discussion

The importance of using adequate amounts of the reagents, and particularly of HCl, in the estimation of saccharated iron oxide cannot be over-emphasized. Established methods for the estimation of serum iron use too little HCl to split this compound completely. The comparatively low figures (about a quarter of the expected values) obtained by Cameron, Bensley, and Wood (1951), who used the method of Barker and Walker (1940) for the estimation of serum iron, are evidently due to the small amounts of HCl added (1 ml. of 1.2% HCl to 2 ml. of plasma).

The present work represents the first comparative study of plasma iron and urinary iron excretion after the intravenous administration of different iron preparations at such high dose levels. It was carried out as part of an investigation into the different mechanisms of toxicity of iron preparations. The investigation by McCance and Widdowson (1938) of urinary and faecal iron excretion after repeated daily intravenous iron and ammonium citrate in man bears little relation to the present work in aim, method of administration, and dosage. It is of interest, however, that both in these and in the present experiments iron excretion was mainly renal, practically no iron being eliminated through the bowel.

The experiments described here demonstrate the wide differences in the rate of disappearance of iron from the plasma after the intravenous administration of different iron preparations. The plasma iron level immediately after injection may correspond to the theoretical value based on the assumption that there is complete and even distribution in plasma. This is so with saccharated iron oxide, ferric glucosate, and "ferric chloride

lactate." The subsequent rate of fall is slow, and is presumably due, in the main, to the activity of the reticulo-endothelial system. With other iron preparations a rapid fall in plasma iron occurs. This may be due to rapid flocculation, as is known to occur with colloidal ferric hydroxide. A third group of preparations show a rapid fall in plasma iron associated with high urinary iron excretion, e.g. iron and ammonium citrate, " ferric chloride caramelate," and " ferric hydroxide ferrous ascorbate."

The three experiments on rabbits with ligated renal vessels gave consistent results and established that the rapid fall of plasma iron after iron and ammonium citrate as compared with saccharated iron oxide was not due merely to greater urinary elimination. The rapid disappearance of iron from the plasma after iron and ammonium citrate and the other preparations which showed a high urinary iron excretion can therefore only partly be accounted for by the quantity of iron in the urine. The estimation of iron in the faeces plus alimentary tract contents has shown that excretion via the alimentary tract must be negligible if it occurs at all. The difference in diffusibility between the different iron preparations, on the other hand, is indeed striking, those with high urinary iron excretion also having the greatest diffusibility.

This interesting correlation between iron preparations in urinary iron excretion and diffusibility does not appear absolute, however, since ferric tartrate and ferronascine, which occupy an intermediate position regarding diffusibility, show rather poor renal elimination. The present diffusibility studies provide ample explanation for the very rapid fall in plasma iron levels which occurs with " ferric hydroxide ferrous ascorbate," " ferric chloride caramelate," and iron and ammonium citrate—a fall which could not be fully accounted for by their high urinary elimination.

Summary

Iron preparations fall into one of three groups. The first includes compounds like saccharated iron oxide, ferric glucosate, and " ferric chloride lactate," which exhibit a slow rate of disappearance from the blood associated with poor urinary excretion. Compounds of the second group, such as colloidal ferric hydroxide, show rapid fall of plasma levels associated with rapid precipitation of the iron and poor urinary excretion. In the third group are such compounds as iron and ammonium citrate, " ferric chloride caramelate," and " ferric hydroxide ferrous ascorbate," which show a rapid fall in plasma iron associated with high urinary excretion.

The highest percentage of urinary excretion was obtained with " ferric hydroxide ferrous ascorbate " (27.4%), the lowest with colloidal ferric hydroxide (0.27%). The three compounds showing the highest renal elimination also possess the highest diffusibility. This accounts for the rapid fall in their plasma levels even after renal ligation.

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A PHARMACOLOGICAL ANALYSIS OF THE MODE OF ACTION OF SEROTONIN (5-HYDROXYTRYPTAMINE) UPON THE GUINEA-PIG ILEUM

BY

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(RECEIVED NOVEMBER 11, 1952)

Serotonin, the substance isolated by Rapport, Green, and Page (1948) from shed blood, was identified with 5-hydroxytryptamine-creatinine sulphate. A close parallelism between the pharmacological properties of serotonin and those of thrombocytin and circulating enteramine was recently established by Reid and Rand (1952) and Erspamer and Asero (1952). Besides the possible intrinsic importance of serotonin as an endogenous principle that may be mobilized in physiological or pathological conditions, serotonin presents interesting problems from a pharmacodynamic point of view the solution of which might help to throw light on questions concerning the mode of action of drugs upon smooth muscle.

When given intravenously, serotonin produces a fall followed by a rise of blood pressure. Atropine appears to abolish the fall and has no effect upon the rise, as shown recently by Page (1952). The action of serotonin upon the smooth muscle of the guinea-pig ileum is strongly influenced by atropine and appears to be parasympathomimetic or cholinergic in nature. Antihistaminic drugs have no action upon this smooth muscle stimulating effect. Tachyphylaxis to serotonin has been noted by Reid and Rand (1952) and by Freyberg *et al.* (1952).

The experiments presented here were undertaken to clarify the mode of action of serotonin upon the guinea-pig gut and especially as an attempt to localize its site of action among the different anatomical structures allegedly responsible for myokinetic drug effects. A preliminary account of this work has already been published (Rocha e Silva, Valle, and Picarelli, 1952).

METHODS AND MATERIALS

The conditions of the assay upon the guinea-pig ileum were the same as described previously (Ambache and Rocha e Silva, 1951). A chamber containing 3.5 ml. of Tyrode solution was used in some preliminary experiments, being replaced by one of 15 ml. capacity in most of the experiments described below. Where not otherwise indicated, the chamber used was the larger one. The drugs were usually added at 1.5 minute intervals; when longer intervals were required these are indicated by a cross (x) in the tracings or in the legends to the figures. After two washings with fresh Tyrode solution, a one minute resting period was allowed for recovery of the muscle. The standard histamine used was a 1 : 2 million solution, calculated as the base, of the dihydrochloride (Eastman Kodak). When not otherwise indicated, the doses of histamine and serotonin were respectively 0.2 of the 1 : 2 million solution and 5 μg. of serotonin. The routine dose of nicotine was 10 μg. of the base. For the sake of brevity, the name of serotonin has been used throughout the paper, although it is well established that the active part of the complex substance is the molecule of the base 5-hydroxytryptamine. All doses indicated in the paper in terms of the creatinine sulphate complex must be divided by 2.3 in order to express them in terms of the base. Acetylcholine was used as a 1 : 1 million solution of the hydrochloride (Roche). Other drugs studied were adrenaline (Parke Davis), pilocarpine hydrochloride, hexamethonium iodide, and *d*-tubocurarine (Intocostrin, Squibb and Sons). The serotonin (creatinine complex with 5-hydroxytryptamine) was obtained through the courtesy of Dr. R. Coghill of Abbott Laboratories, North Chicago, U.S.A.

RESULTS

Tachyphylaxis to Serotonin.—Small doses (4 to 5 μg.) of serotonin added to the bath containing a piece of guinea-pig ileum cause responses that are reproducible without any sign of tachyphylaxis. The responses to doses as low as 10 μg. of serotonin, however, cannot be kept at a constant level if the interval between additions is 1 or 2 minutes. If the interval between additions of 10 μg. of serotonin is increased to 3 minutes, reproducible responses can be obtained, as shown in Fig. 1. By keeping this interval between additions, and by using doses smaller than 20 μg., a dose-response relationship

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may be found and a 2×2 bioassay can be designed (Valle and Picarelli, 1952).

The effect obtained with small doses of serotonin is similar to that observed after small, stimulating doses of nicotine, and is distinguished by minor details from that produced by histamine or acetylcholine. After an almost instantaneous rise of tonus the muscle shows a certain tendency to return to a lower tonus, as soon as the maximum is attained, as contrasted with the sustained tonus observed with histamine, as shown in Figs. 1 and 2.

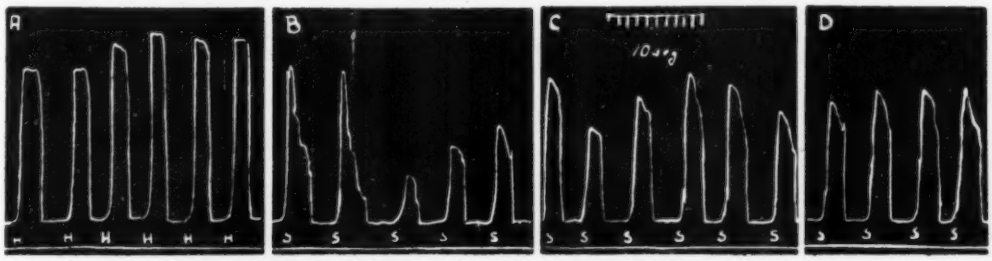

FIG. 1.—Guinea-pig ileum. Repeated additions of histamine 0.1 μg. (H), in panel A; of 10 μg. serotonin at 1 minute intervals in B; the same at 2 minute intervals in C, and at 3 minute intervals in D.

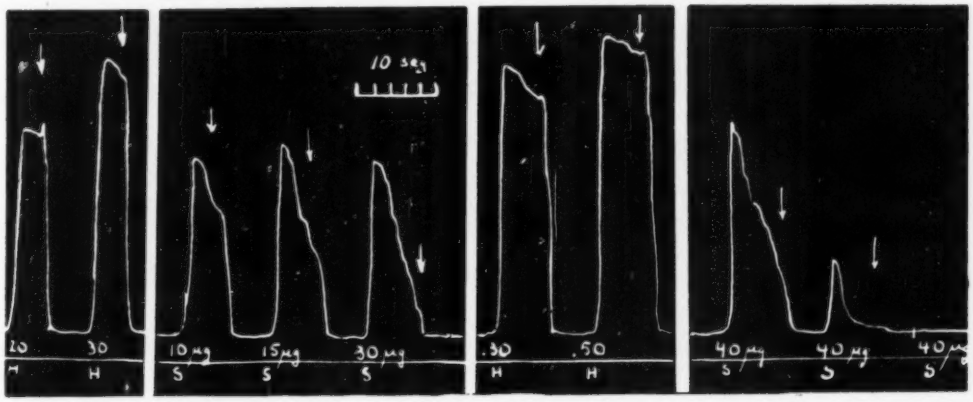

FIG. 2.—Guinea-pig ileum. Comparison between the additions of increasing doses of histamine (H) and serotonin (S). The doses of histamine are indicated as ml. of 10^{-6} solution.

If the doses of serotonin are increased to 15, 30, or 40 μg. the responses are no longer quantitative and a tendency to partial or total tachyphylaxis is seen, as shown in Fig. 2. With doses of 40 μg. of serotonin, added to a bath of 15 ml. capacity, we can no longer maintain a steady level of responses, as indicated in Fig. 3, although histamine and acetylcholine still elicit reproducible responses. This difference of behaviour of the gut toward serotonin, as compared with histamine and acetylcholine, indicates that serotonin has a different locus of action. It is clear that serotonin acts upon other than histamine receptors, since antihistaminic drugs have no effect upon it. But, since atropine blocks serotonin, it is possible that serotonin acts primarily upon acetylcholine receptors. The experiments presented in Fig. 3, however, which show a tachyphylactic effect to serotonin but not to acetylcholine, indicate that serotonin affects distinct receptor structures.

Large doses of serotonin (50 to 200 μg. or more) produce a quick and powerful contraction followed by a sharp fall of tonus. Repeating the addition of the same large dose will produce no effect and the muscle remains refractory to the addition of a small dose of serotonin for quite a long interval of time, as shown in Fig. 4*a*. Recovery to such

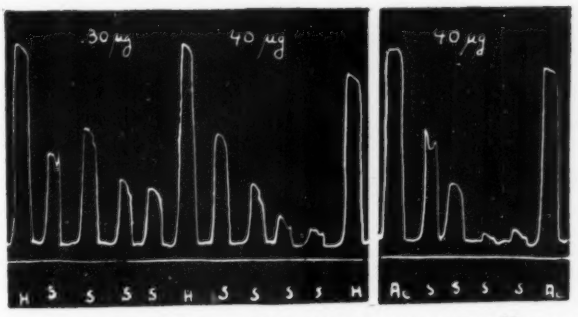

FIG. 3.—Guinea-pig ileum. Decrease in the responses to 30 μg. or 40 μg. of serotonin does not affect the responses to 0.1 μg. of histamine (H) and 0.15 μg. of acetylcholine (Ac).

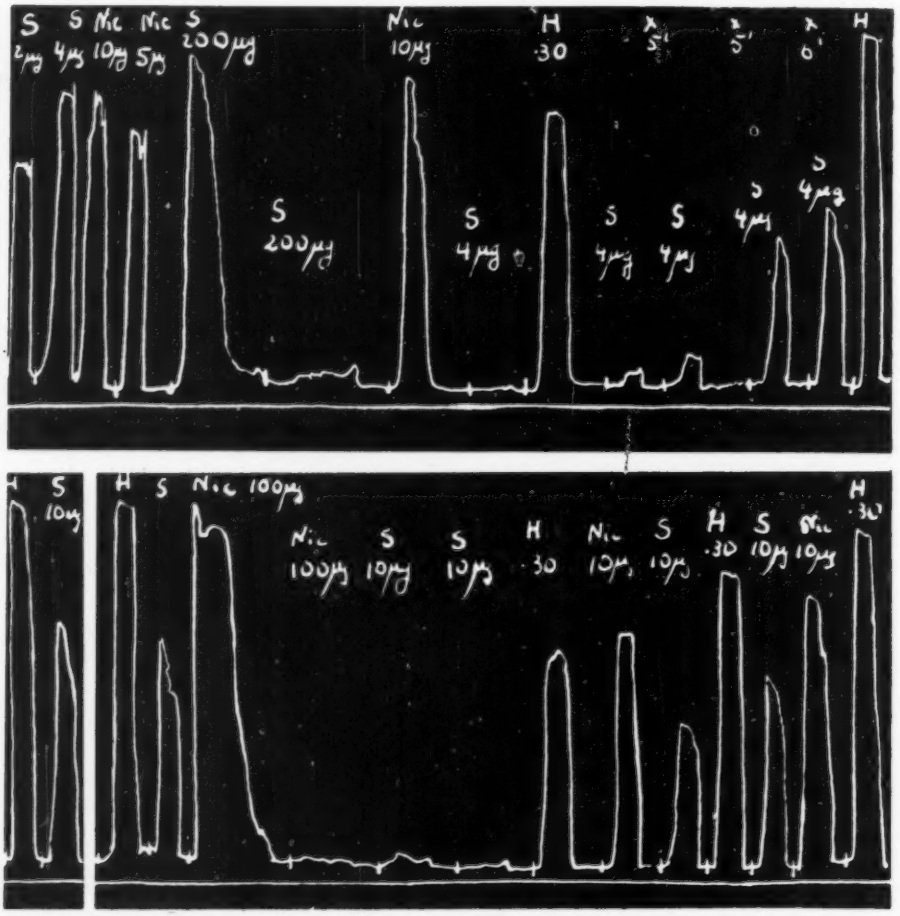

FIG. 4.—(*a*) Addition of 200 μg. of serotonin (S) to the bath produces a quick response followed by an immediate return to the normal level of tonus; the muscle will not react to the same dose or smaller doses of serotonin, although the reactions to nicotine and histamine remain unimpaired. (x) indicates stoppage of the drum for the times indicated. (*b*) Similar experiment with two additions of 100 μg. of nicotine. Note that during refractoriness to smaller doses of nicotine the response to 10 μg. of serotonin is abolished and the response to histamine (H) is strongly depressed. Doses of histamine indicated as ml. of 0.5×10^{-6} solution.

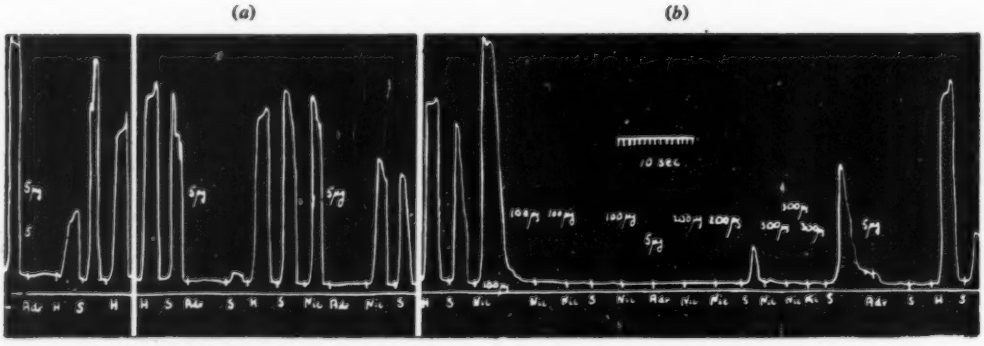

FIG. 5.—(*a*) The responses of the guinea-pig gut to histamine or serotonin are strongly depressed by previous treatment with 5 μg. of adrenaline. Note that 0.1 μg. histamine (H) was less affected than 5 μg. of serotonin (S) by the previous treatment of the gut with adrenaline; nicotine (Nic) was even less affected. (*b*) After three additions of 100 μg. of nicotine the response to serotonin is abolished; after further treatment with 100 μg. to 200 μg. of nicotine, the muscle begins to respond to serotonin, while completely refractory to nicotine itself; this "escape" from the inhibitory effect of nicotine is better seen after three further additions of 300 μg. of nicotine; it is interesting to note that adrenaline (5 μg.) still depressed the response to serotonin.

doses of serotonin takes a longer or shorter time to be complete, depending upon the size of the paralysing dose applied at the beginning. Tachyphylaxis toward serotonin is, therefore, temporary and can be distinguished from other types of tachyphylaxis, such as that observed after successive additions of certain bacterial toxins or animal venoms, in which the muscle remains indefinitely refractory after a first of two additions of any dose of the stimulating material. It can also be distinguished from the tachyphylaxis to antigen in sensitized guinea-pig gut (*in vitro* anaphylaxis), where also, after a first contact with the antigen, the muscle remains indefinitely refractory to the addition of the same dose.

Tachyphylaxis to paralysing doses of serotonin has some features similar to that obtained with large doses of nicotine (as shown in Fig. 4*b*) in that it is very quick to develop (while the drug is still in the bath), is reversible, and is not so unspecific as that seen after large doses of other spasmogenic agents, such as histamine, acetylcholine, or pilocarpine (Cantoni and Eastmann's effect). It can, however, be distinguished from the similar nicotinic effect by not altering appreciably the responses to other drugs such as histamine or acetylcholine. This difference, though slight, appeared of importance in interpreting some results that will be presented hereafter.

Effect of Nicotine upon Serotonin Action.—Small to moderate doses of nicotine (20 to 40 μg.) can depress the gut to serotonin. A full depression of serotonin by nicotine is observed with moderate paralysing doses (50 to 200 μg.), as shown in Figs. 4*b*, 5, and 6. When such doses are added to the chamber, a powerful contraction followed by a quick return to normal or subnormal tonus is seen. If the muscle is then tested with: (*a*) serotonin (4 to 10 μg.); (*b*) histamine; (*c*) acetylcholine; (*d*) nicotine (10 to 15 μg.); (*e*) bradykinin; (*f*) pilocarpine and (*g*) eserine, it is shown to be completely blocked toward *a* and *d*, moderately blocked to *b*, *c*, *e*, and *f*, and still reactive to *g*.

The complete block toward stimulating doses of serotonin produced by moderate paralysing doses of nicotine might induce one to assume that serotonin also acts upon the ganglion cells. Nevertheless, its site of action must be somewhat different from that of nicotine, since a paralysing dose of serotonin does not affect the responses to stimulating doses of nicotine.

The idea that serotonin acts upon the ganglion cells was, however, abandoned when we verified that the inhibitory effect produced by moderate paralysing doses of nicotine could not be obtained after applying large paralysing doses (300 to 400 μg.), even when these were repeatedly applied

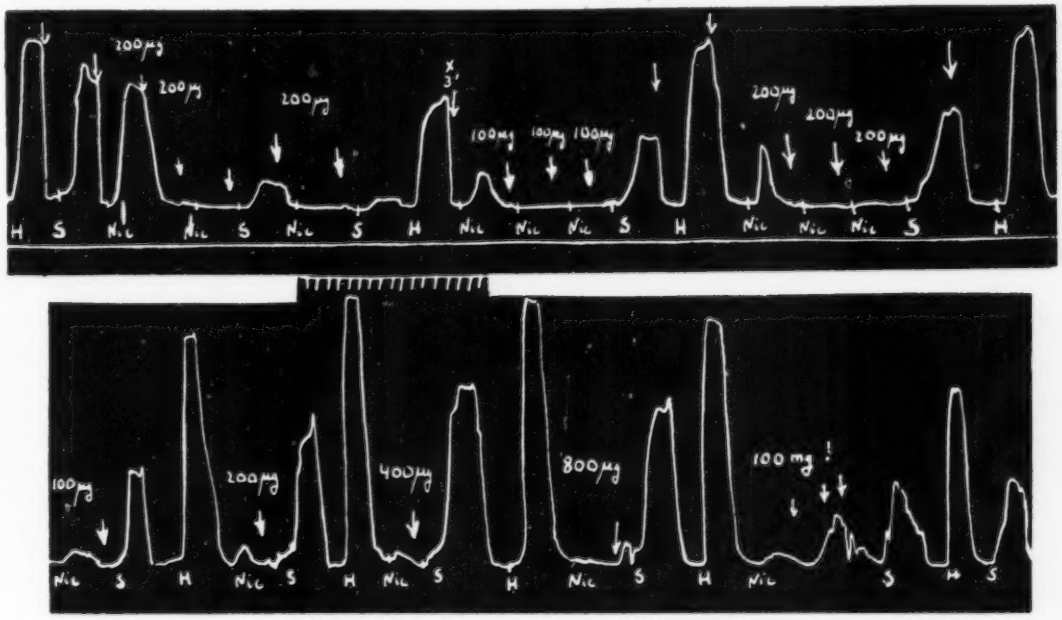

FIG. 6.—(*a*) "Escape" from inhibition by nicotine (Nic). After a strong inhibition of 5 μg. of serotonin (S) and 0.1 μg. of histamine (H) produced by 2 or 3 additions of 200 μg. of nicotine, the muscle recovers its full sensitivity to both agents after the further addition of increasing doses of nicotine. (*b*) Same piece of ileum. The responses to histamine or serotonin become progressively stronger as the dose of nicotine increases. The arrows indicate washings with fresh Tyrode solution.

to the intestine, as shown in Fig. 6*a* and *b*. This " escape " from inhibition by nicotine could be observed by repeatedly adding such moderate paralysing doses as 100 to 200 μg. of nicotine, as shown in Fig. 7. A speedier return of the full responsiveness to serotonin could be seen after the addition of a few large doses of nicotine (300 to 400 μg.). It is interesting to note that large doses of nicotine may produce a similar " escape " from the partial inhibition, by smaller doses of nicotine,

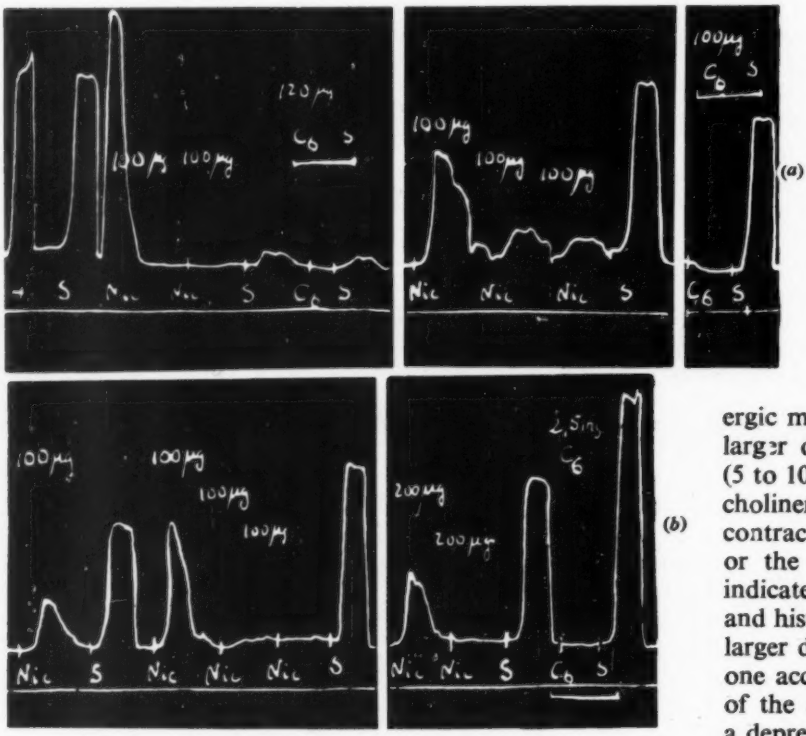

FIG. 7.—(*a*) Hexamethonium (C_6) added during refractoriness to 5 μg. of serotonin (S) produced by nicotine (Nic) will not change the sensitivity of the muscle to serotonin. After repeating the additions of nicotine, the muscle " escapes " from inhibition and responds fully to serotonin; hexamethonium added again before serotonin and kept in the bath does not affect the size of the response. (*b*) After repeated treatments with 100 to 200 μg. of nicotine the full sensitivity to serotonin is restored and hexamethonium potentiates serotonin action.

of the effects of histamine, acetylcholine, or bradykinin (Ambache and Rocha e Silva, 1951; Rocha e Silva, 1952). In Fig. 6*a* and *b*, a partial inhibition of the histamine effect by repeated addition of 200 μg. of nicotine was followed by a clear-cut " escape " after further treatment with 100 to 800 μg. of nicotine.

The foregoing results indicate that the depression caused by moderate paralysing doses of nicotine is not due to the ganglionic paralysis itself, since this would be even greater with the larger doses of nicotine. They also clearly demonstrate that the depression produced by nicotine could not result from any unspecific depressant effect upon the muscle itself, as suggested by Emmelin and Feldberg (1947) and Feldberg (1950), since this kind of depression too would obviously be greater with the larger doses. It is true that massive doses of nicotine, such as 800 μg., can sometimes depress the effect of serotonin or histamine, as shown in Fig. 6*b*, but this effect is slight compared with the strong depression produced by moderate (50 to 100 μg.) doses of nicotine.

Instead of assuming a direct depression of the contractility of the muscle by such moderate doses of nicotine, we are more prone to accept the possibility that the inhibitory effect is due to the release of minute amounts of a mediator from adrenergic fibres. As with the parasympathetic ganglion apparatus, larger doses of nicotine (300–400 μg.) would exhaust the system. According to the experiments presented above, the adrenergic mechanism would require somewhat larger doses of nicotine, since a few μg. (5 to 10 μg.) apparently act only upon the cholinergic ganglion system, producing a contraction without affecting the histamine or the serotonin added afterwards. As indicated above, a depression of serotonin and histamine is seen only after somewhat larger doses of nicotine (30 to 50 μg.). If one accepts the hypothesis of a discharge of the adrenergic system to explain such a depression, these doses are probably the threshold ones for nicotine to affect the inhibitory fibres of the intestinal wall. Abolition of the depression of serotonin or histamine (" escape from nicotine ") can be obtained by adding doses of nicotine that are twice or three times larger than those necessary to paralyse the parasympathetic ganglion system. We may therefore assume that the concentrations of nicotine which affect the intramural adrenergic system are two to three times higher than those which act upon the parasympathetic ganglion cells.

Effect of Adrenaline upon Serotonin Action.—In order to substantiate the hypothesis of a discharge of the adrenergic system by moderately paralysing doses (50 to 100 μg.) of nicotine, to explain the depression of the responses to serotonin and other

drugs such as histamine, acetylcholine, and bradykinin, we have tried to correlate the depression produced by nicotine with that observed by adding adrenaline. A perfect parallelism would not, of course, be expected, since we do not know whether adrenaline or noradrenaline, or a mixture of both, would be released by the stimulating effect of nicotine. Thus a commercial adrenaline preparation "Adrenalin," Parke Davis) was judged likely to give as good a result as the use of pure adrenaline or noradrenaline.

Extremely small concentrations of adrenaline (0.07 μg./ml.) in the bath depressed the effect of histamine and serotonin. The latter was found to be more influenced than the former by such small concentrations. With bradykinin and histamine we have found, in some preparations, that both are influenced in the same way by adrenaline and by nicotine. It is of interest to note that on adding increasing doses of nicotine, the muscle "escaped" from inhibition toward bradykinin as it usually does toward serotonin and histamine.

The "escape" to nicotine appears to be a general phenomenon and can probably be explained by a diphasic effect upon the adrenergic system. In a few preparations the inhibition toward histamine or serotonin can be obtained when small doses of nicotine are added, but a definite depression is usually seen after doses around 50 to 100 μg. of nicotine. Even with such moderate doses of nicotine, if repeatedly added to the bath, the "escape" to the inhibitory effect of nicotine may be noticeable; but a quicker "escape" can only be seen if the doses of nicotine are increased beyond the 300 or 400 μg. level. A direct "toxic" effect of the alkaloid upon the muscle fibres is only obtained with much higher doses, far above the mg. level.

Effect of Decamethonium, Hexamethonium, and d-Tubocurarine on Serotonin Action.—The peristaltic reflex is abolished by hexamethonium (C6) as shown by Paton and Zaimis (1949), and this effect is due to a paralysing action of hexamethonium upon the parasympathetic ganglion cells of the mammalian intestine. Feldberg (1951) considered hexamethonium the most specific ganglion inhibitor, as far as the guinea-pig gut is concerned.

Decamethonium (C10) displays very slight or no effect as a ganglion blocking agent when assayed upon the guinea-pig gut. According to Feldberg (1951), *d*-tubocurarine has strong atropine-like activity, since it depresses the response to acetylcholine as well as that to nicotine.

Hexamethonium has no inhibitory effect upon the contraction elicited by serotonin upon the guinea-pig ileum, as is shown in Fig. 8*a* and *b*. In most instances the addition of 50 to 100 μg. of hexamethonium tended to enhance the effect produced by 10 μg. of serotonin. The results obtained with C6 indicate very clearly that serotonin does not act through stimulation of the parasympathetic ganglion cells. This agrees with the fact, presented above, that very large doses of nicotine, such as completely block the parasympathetic ganglion system, have no action upon serotonin. The enhancement of the serotonin effect by C6 makes it interesting to recall that ganglion blocking

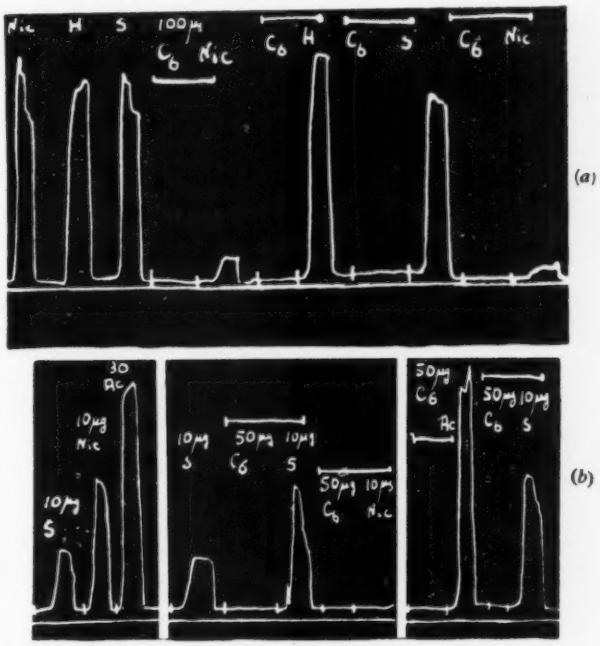

FIG. 8.—Hexamethonium (C_6) in concentrations that almost abolished the nicotine responses had no effect upon 0.1 μg. histamine (H) or 5 μg. serotonin (S) or 0.3 ml. 10^{-6} acetylcholine (Ac), though it had sometimes a potentiating effect upon serotonin (middle panel of *b*). The white horizontal lines indicate that both drugs were kept in the bath.

agents have been described as potentiating the effects of other spasmogenic drugs. Thus hexamethonium and tetraethylammonium sometimes potentiate the effect of histamine (Feldberg, 1951, and Collins, 1948), while *d*-tubocurarine has a potentiating effect upon histamine (Rocha e Silva and Schild, 1949, and Feldberg, 1951). This potentiating effect was interpreted by Feldberg (1951) as due to an increase of excitability of the muscle fibres after blockade of the ganglion cells. The effect of C6 upon serotonin, as shown in Fig. 7*b* and Fig. 8*b*, can likewise be interpreted in the same way.

Decamethonium (C10), when added in doses as high as 100 μg., had no effect upon the serotonin induced contraction (Fig. 9*a*). The inhibition by

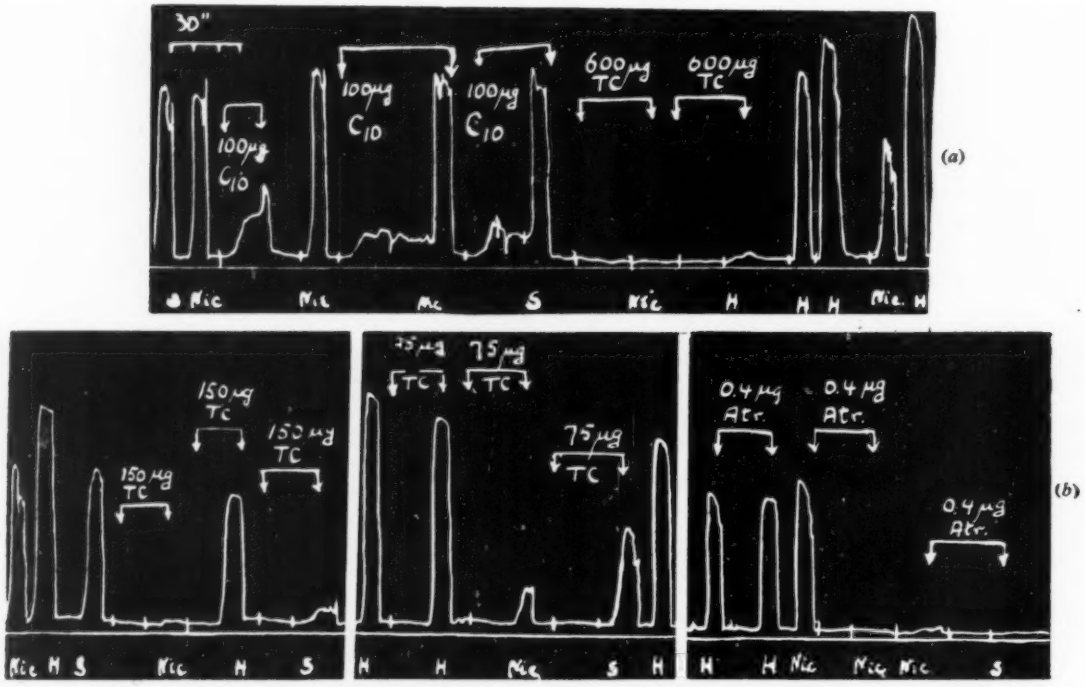

FIG. 9.—(*a*) Decamethonium (C_{10}) in a dose of 100 μg. had no effect upon 10 μg. nicotine (Nic) and 5 μg. serotonin (S). High doses (0.6 mg./15 ml.) of *d*-tubocurarine totally blocked the stimulating effect of nicotine and histamine. (*b*) Smaller doses of *d*-tubocurarine totally (150 μg./15 ml.) or partially (75 μg./15 ml.) inhibited the response to nicotine and serotonin, the blocking effect being greater upon nicotine and much less so upon histamine. A small dose of atropine (0.4 μg.) entirely blocked nicotine and serotonin and had no effect upon histamine.

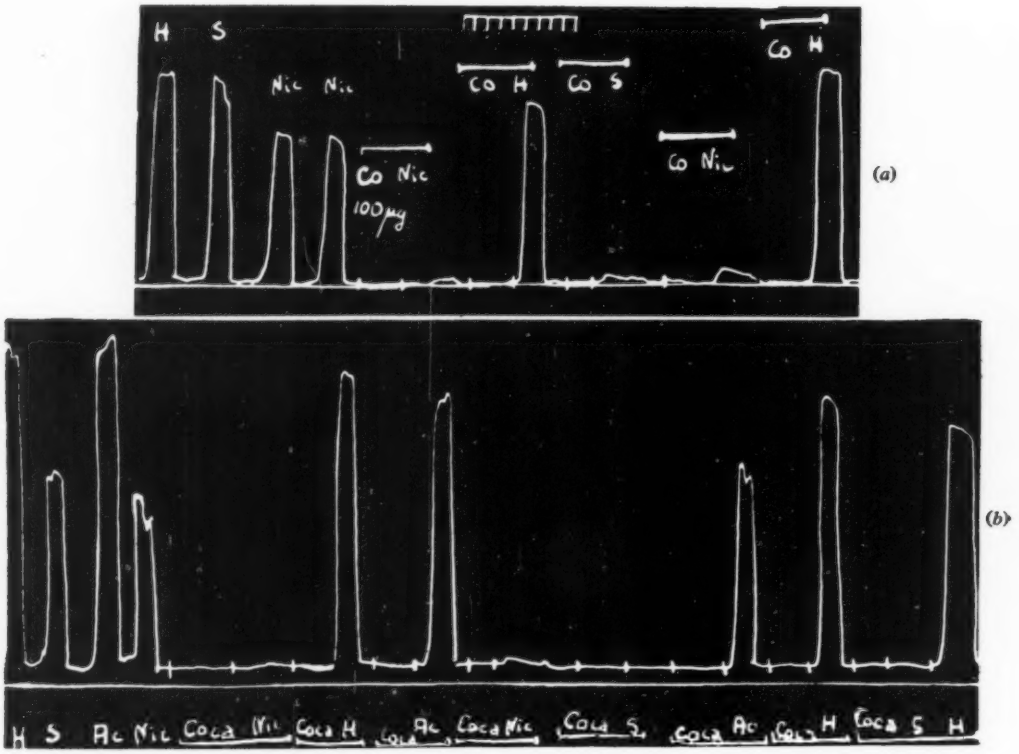

FIG. 10.—(*a*) and (*b*). Cocaine (Co), in concentrations (100 μg./15 ml.) that had no effect upon 0.1 μg. histamine (H) or 0.15 μg. of acetylcholine (Ac), completely abolished the responses to 10 μg. nicotine (Nic) or 5 μg. serotonin (S).

d-tubocurarine, shown in Fig. 9*a* and *b*, might be accounted for by its atropine-like activity, demonstrated by Feldberg (1951).

Effect of Cocaine upon Serotonin Action.—According to Feldberg and Lin (1949, 1950) it is possible, with a suitable dose of cocaine, to paralyse the nicotine stimulating action, without impairing the effect of histamine or acetylcholine. Doses of cocaine below 0.4 mg./ml. are usually able to block completely the stimulating effect produced by 10 μg. of nicotine and have no effect upon other drugs supposed to act directly upon the muscle (Fig. 10*a* and *b*). With serotonin we have found that such a minute dose of cocaine as 10 μg. in a 15 ml. bath will inhibit the spasmogenic effect of 10 μg. of serotonin, as shown in Fig. 11. An inhibition of the histamine or acetylcholine effects can only be obtained with doses 50 to 150 times stronger, and even then the blockade is not complete.

The inhibitory effect of threshold doses of cocaine upon serotonin gave us the key to explain the locus of action of the latter drug upon the gut. Since cocaine, in concentrations that will not affect histamine or acetylcholine, is supposed to act by blocking the transmitting fibres of the myenteric plexus, only two possibilities are left to explain inhibition of the action of serotonin upon the gut: (*a*) the pre-ganglionic and (*b*) the post-ganglionic parasympathetic fibres. A stimulus upon *a* would need, of course, a free path through the ganglion cells; but, as we have shown in the above paragraphs, serotonin acts upon the gut even when the ganglion cells have been completely blocked either by large doses of nicotine or by hexamethonium. Apparently, therefore, the only indirect point of attack for serotonin would be the post-ganglionic fibres that can be blocked by cocaine (see diagram, Fig. 12).

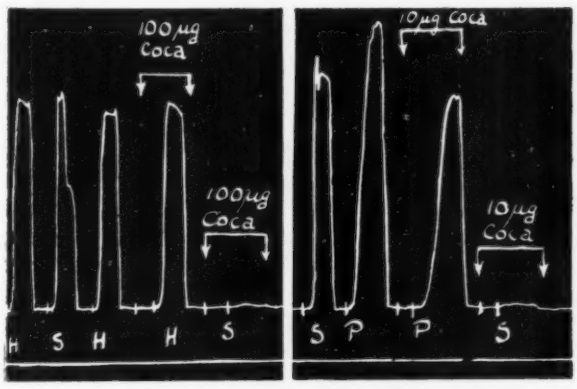

FIG. 11.—100 μg. of cocaine (Coca) had no effect upon 0.1 μg. histamine (H), but completely blocked the effect of 5 μg. serotonin (S). Inhibition of the serotonin action was observed with doses as small as 10 μg. of cocaine per 15 ml. A partial block of pilocarpine (P) was observed with 10 μg. of cocaine added to the bath (15 ml.).

DISCUSSION

In any attempt to localize the site of action of a drug upon such a complicated anatomical structure as the gut of vertebrates, we have to be aware of the fact that all evidence is indirect, since it is impossible to isolate any single motor unit as can be done with striated muscle (Fisher, 1944). There are, however, a few data that can provide solid ground on which to establish a theory to account for the experimental results presented above. There is no question, for example, that the efferent nerve fibres to the mammalian small intestine come from the two main sections of the autonomic nervous system—the parasympathetic and the sympathetic outflow. There is also enough histological evidence to show that most of the ganglion cells of the myenteric plexus of Auerbach and Meissner belong to the parasympathetic system, while the possibility that some of the adrenergic terminal fibres come from ganglion cells of the myenteric plexus is still open. Ambache and Edwards (1951) have recently provided fresh evidence of the presence of synapses along the sympathetic fibres to the cat intestine. But, whatever be the final answer to that question, one has to consider a system of inhibitory fibres in close connection with the smooth muscle effectors. A third class of nerve fibres is constituted by the so-called " terminal reticulum," a tangled system of argyrophil neurofibrils around the ganglion cells and in close association with the so-called Cajal cells. A discussion of the whole problem can be found in Fisher (1944), Feldberg and Lin (1949), Ambache (1946), and Ambache and Edwards (1951). The diagram (Fig. 12) gives a schematic view of the possibilities for the different loci of action of drugs upon the guinea-pig gut. The structures have been drawn separately for the sake of simplicity, but it seems probable that the " degree of entanglement " is higher than is represented in the diagram.

In such conditions, we have to rely upon the indirect information obtained by applying inhibitors of several kinds—antihistaminics, atropine, ganglion blocking agents (decamethonium, hexamethonium, nicotine, and *d*-tubocurarine), local anaesthetics and so forth—to define the mode of action of any substance upon the gut. The evidence presented above strongly indicates that serotonin might be considered a specific stimulant of the post-ganglionic cholinergic fibres. Our knowledge of the physiological significance of the so-called " ter-

minal reticulum" is scanty, but if it be found that such fibres are cholinergic in character they might well constitute a site of action for an indirectly working drug such as serotonin. That serotonin has receptors of its own, distinct from those for histamine, acetylcholine, pilocarpine, bradykinin, or nicotine, is suggested by the following facts: (*a*) antihistaminics will not abolish the effect of serotonin; (*b*) when the muscle is made refractory to a higher dose of nicotine, and its ganglion cells are thus paralysed, serotonin will act upon the gut better than when the latter has been paralysed by a moderate dose of nicotine; (*c*) when the muscle is made refractory to serotonin by applying a large paralysing dose of this substance, it still responds to histamine, nicotine, acetylcholine, and pilocarpine; (*d*) hexamethonium completely blocks the action of nicotine, yet rather potentiates the effect of serotonin; (*e*) cocaine, which in the concentrations used will not affect histamine or acetylcholine, entirely blocks the action of serotonin upon the gut. Since serotonin apparently does not work directly upon the muscle fibres of the intestine, or upon the ganglion cells, its stimulating effect can properly be ascribed to a discharge, followed by temporary exhaustion, of the post-ganglionic cholinergic fibres.

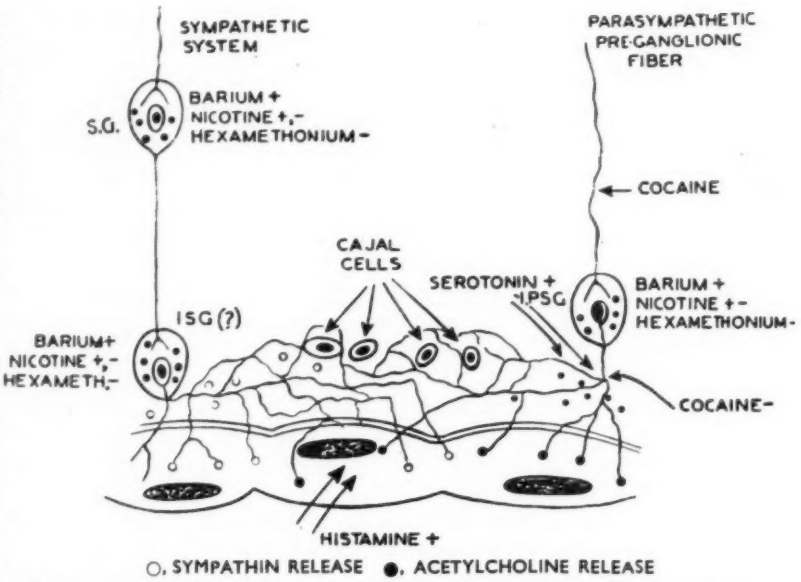

FIG. 12.—Schematic picture of the possible loci of drug action upon the guinea-pig ileum. ISG, intramural sympathetic ganglion (hypothetical); IPSG, intramural parasympathetic ganglion cell; ○, sympathin release; ●, acetylcholine release.

A point of interest to be discussed is the mechanism of the "escape" to the paralysing dose of nicotine. Small, paralysing doses of nicotine (50 to 100 μg.) strongly depress or abolish the effect of serotonin upon the gut. Other drugs, such as histamine, acetylcholine, and bradykinin, are also depressed by such doses of nicotine, although as with bradykinin this depression is seen irregularly, as indicated by Ambache and Rocha e Silva (1951). Feldberg (1950) assumed that nicotine in such doses would depress unspecifically the smooth muscle fibres to any gut stimulating drug. On the contrary, Ambache (1946) pointed out that this effect of nicotine in depressing histamine or acetylcholine would indicate that such drugs act at least partially through the ganglion systems. That neither view is correct was first shown by Ambache and Rocha e Silva (1951) on the basis of the fact that the depression produced by nicotine is stronger with moderate doses (stimulating and paralysing doses), and that, if the addition of the same dose was many times repeated or higher doses (200 μg. to 1 mg.) were used, the inhibition was considerably reduced or abolished. To explain the depression produced by moderate (stimulating and paralysing) doses of nicotine, Ambache and Rocha e Silva (1951) assumed, in accordance with Emmelin and Feldberg (1947), that partial refractoriness of the muscle cells might be due to Cantoni and Eastmann's effect—that is, to exhaustion of energy-rich metabolites after the maximal contraction produced by such doses of nicotine. The absence of depression after the higher (paralysing only) doses of nicotine might result from the contraction being of such short duration—or being absent altogether—that Cantoni and Eastmann's effect could not come into play. This explanation might also hold for the total inhibition of serotonin by moderate (stimulating and paralysing) doses of nicotine; while its "escape" from nicotine inhibition might be accounted for by the fact that if the muscle is kept for a long time under the paralysing action of nicotine no metabolites are consumed so that, again, Cantoni and Eastmann's effect is absent.

However, an alternative explanation has been presented in this paper, based on the possibility of nicotine discharging and paralysing the adrenergic inhibitory fibres. This requires only the assumption that the range of doses in which nicotine acts upon the parasympathetic ganglion system is lower than that in which it acts upon the inhibitory intramural

system. That nicotine can stimulate the inhibitory system was demonstrated by Ambache and Edwards (1951) in the atropinized intestine of the cat. That this effect might result from a release of adrenaline or noradrenaline is a strong possibility. In the depression produced by nicotine upon the effects of several drugs a similar mechanism might operate. If one assumes that moderate doses (50 to 100 μg.) of nicotine discharge the adrenergic fibres to the smooth muscle with liberation of minute amounts of a sympathomimetic mediator (adrenaline or noradrenaline, or both) the fall in tonus and also the unspecific inhibition toward serotonin, histamine, acetylcholine, pilocarpine, etc., might be due to the effect of the liberated mediator. If it be so that nicotine discharges these fibres, or the ganglion cells interposed in their pathways, one might expect that large doses (up to 200 to 400 μg.) of nicotine would also paralyse this system, in the same way that smaller doses will discharge and paralyse the cholinergic ganglion system. This view agrees with the observation by Feldberg and Lin (1949) that moderately paralysing doses of nicotine produce a fall of tonus of the rabbit intestine, from which inhibition the muscle recovers, even after having been submitted to the continuous action of nicotine.

Summary

1. The mode of action of serotonin (complex of creatinine+5-hydroxytryptamine) was studied upon the isolated guinea-pig ileum. The stimulating effect of serotonin on the gut was blocked by atropine and appeared, therefore, to be cholinergic in nature. Tachyphylaxis could be observed with small doses (10 μg./15 ml.) if they were added at intervals of less than 3 minutes.

2. Total, though transitory, tachyphylaxis was observed after repeated additions of 40 μg./15 ml. of serotonin. With higher doses, a quick contraction followed by spontaneous return to the normal tonus (while the drug still was in the bath) was observed. For a certain interval of time thereafter the muscle remained refractory to smaller doses of serotonin, but still reacted to other drugs, including nicotine. Sensitivity to small doses of serotonin progressively reappeared.

3. Moderate paralysing doses (50 to 100 μg.) of nicotine depressed or abolished the responses to serotonin and also depressed the responses to histamine, acetylcholine, and bradykinin. If much higher doses (200 to 800 μg.) of nicotine were added, the muscle "escaped" from inhibition by nicotine, responding again to serotonin and increasing in sensitivity to the other drugs, although continuing to be irresponsive to nicotine itself.

4. Decamethonium and hexamethonium had no inhibitory effect upon serotonin action. In some experiments, hexamethonium even potentiated serotonin action. *d*-Tubocurarine in concentrations that had no effect upon histamine depressed the effects produced by serotonin or nicotine.

5. The action of serotonin was completely blocked by cocaine in doses (10 to 100 μg.) that did not affect histamine or acetylcholine.

5. From these observations the provisional conclusion is drawn that serotonin acts upon the post-ganglionic cholinergic fibres of the intramural nervous system of the guinea-pig ileum.

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Addendum

As indicated in Fig. 9*b*, a dose of atropine of 0.4 μg. in a 15 ml. bath abolished the effect of 5 μg. of serotonin upon the guinea-pig ileum. Under the conditions of our laboratory (São Paulo), using a Tyrode solution of *p*H 7.4, this dose of atropine strongly depressed or abolished the action of small doses of serotonin. Since, in recent papers, Feldberg and Toh (1953) and Gaddum

(1953) claim that atropine does not influence serotonin, we have made further observations on the action of atropine upon serotonin. The conditions of the experiments were different and were those prevailing at the Department of Physiology of the Caroline Institute in Stockholm (3 ml. intestinal bath and a somewhat more alkaline Tyrode, *p*H 8.2). Doses of atropine as small as 0.1 μg./3 ml. strongly depressed (80 to 90% reduction in the size of the response) the effect of 1 to 5 μg. of serotonin; a dose of 0.2 μg./3 ml. almost completely abolished the response to 1 μg. of serotonin. The different results described by the British authors might be due to the use of a more alkaline Tyrode solution, for Dr. Edlund from Uppsala has found that a more acid Tyrode solution (*p*H 6.8) increases considerably the sensitivity of the gut to the inhibitory action of atropine (personal communication).

In some experiments, especially when the gut was very sensitive to histamine, the effect of the latter was also influenced by such concentrations of atropine; but the depression of the histamine effect was much less (25 to 30% reduction) than was the inhibitory effect upon serotonin (90% reduction). But, even in preparations where the histamine effect was not influenced, serotonin was strongly depressed by 0.2 μg./3 ml. of atropine sulphate. Doses of atropine that reduced to 10% or to 0 the action of serotonin had not the slightest effect upon bradykinin (5 to 10 μg. of a preparation containing 8 units per mg.). The effect of substance P is also not affected by such doses of atropine, as shown recently by Pernow (1953). We cannot therefore ascribe to a direct effect of atropine upon the smooth muscle fibres the depression or abolition of the serotonin response. The doses of atropine required to affect that response to serotonin are at least 10 times larger than the threshold doses to abolish the effect of 0.1 μg. of acetylcholine; but if the comparison is made with nicotine as the spasmogenic agent the differences are much smaller. In one experiment upon the same ileum, 0.1 μg./3 ml. of atropine reduced to 15% the effect of 5 μg. of serotonin and to 7% the effect of nicotine. Half of that dose of atropine (0.05 μg./3 ml.) had practically no effect upon 5 μg. of serotonin or 5 μg. of nicotine, although it abolished completely the effect of 0.1 μg. of acetylcholine.

As regards the specificity of the atropine effect, we have analysed the recovery of the gut toward serotonin and acetylcholine, after a 1 minute contact of 0.1 and 0.2 μg./3 ml. of atropine, testing the muscle every 1.5 minute by alternating the additions of serotonin (5 μg.) and acetylcholine (0.1 μg.). The recovery to both drugs followed a parallel curve, essentially similar to that described by Rocha e Silva (1950) for specific antagonists such as antihistaminic, antispasmodic, and atropine-like substances.

I am much indebted to Professor U. S. von Euler for kind advice and for giving me facilities to make these observations at the Caroline Institute, and to Dr. B. Pernow for help in performing the experiments.

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INHIBITION OF SMOOTH MUSCLE CONTRACTILITY BY INDOLE AND SOME INDOLE COMPOUNDS

BY

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(RECEIVED JANUARY 15, 1953)

Among the indole compounds arising in bacterial decomposition of tryptophane, those having the strongest effect on muscle contractility seem to be indole, skatole, and β-indolylethylamine. Their pharmacology has been studied for some time, but only qualitative, often contradictory results have been reported. Thus, whereas indolylethylamine stimulates smooth muscle (Guggenheim and Löffler, 1916), indole and skatole inhibit the contractility of skeletal, heart or smooth muscle (Danilewsky, 1908a, b; Guggenheim and Löffler, 1916; Waddell, 1927; Yanai, 1935; Garcia Blanco, del Castillo, and Rodeles, 1941; and Ets and Feinberg, 1942), although in a few instances they can also stimulate it (Danilewsky, 1908b; Biebl, 1929; and Ets and Feinberg, 1942). The action of indole, skatole, indolylacetate, and L-tryptophane on striated muscle sensitivity to potassium ions and acetylcholine has been more recently studied by Torda and Wolff (1945). According to their observations, indole increases the response of the frog rectus abdominis to acetylcholine, whereas indole, skatole, indolylacetate, and L-tryptophane increase the response to potassium ions.

The main part of this study has been devoted to an analysis of the effect of the indole compounds on the response of guinea-pig ileum to acetylcholine, potassium ions, and histamine. Experiments have also been carried out with duodenum, colon, uterus, and striated muscle preparations. In the latter we have entirely confirmed Torda and Wolff's findings. With smooth muscle organs we find that indole compounds produce the opposite effect: they strongly antagonize the action of acetylcholine and other muscle stimulants. A preliminary account of this work has been given elsewhere (Izquierdo and Stoppani, 1950).

METHODS

Muscle Preparations.—Guinea-pig ileum or colon, rat or mouse colon, rabbit duodenum or rat uterus were washed and set up in the bath, at 37° C., filled with 20 ml. Tyrode fluid (0.8 g. NaCl; 0.02 g. KCl; 0.01 g. $CaCl_2$; 0.1 g. $NaHCO_3$; 0.01 g. $MgCl_2$; 0.005 g. NaH_2PO_4; 0.1 g. glucose and water up to 100 ml.) through which was bubbled a mixture of O_2 with 5% CO_2. The contractions were recorded on a kymograph with an isotonic lever 14 cm. long (magnification: ×4). Responses in the presence of indole compounds were intercalated between responses to the stimulant alone. After each contraction the bath fluid was renewed three times and the organ left at rest for 10 minutes until the next contraction was elicited. Unless stated otherwise, the indole compounds were added immediately after the last washing, remaining therefore 10 minutes in contact with the organ before the subsequent addition of stimulant. The experiments with striated muscle were carried out at room temperature (20°–28° C.) following Chang and Gaddum's (1933) technique. The rectus anterior abdominis of *Leptodactylus ocellatus* (L.) Gir. freshly removed from the animal, or kept for 12 hours in Ringer solution with eserine 10^-, was set up in a bath, as above. For these experiments Ringer solution was used (0.6 g. NaCl, 0.01 g. KCl, 0.01 g. $CaCl_2$, 0.01 g. $NaHCO_3$ and water up to 100 ml.).

Reagents.—Indole, skatole, indolylacetic, and β-indolylpropionic acids, β-indolylethylamine, L-tryptophane, and histamine hydrochloride were obtained from E. Kodak Co.; potassium indoxylsulphate (indican) from T. Schurchardt; and acetylcholine from Hoffman-La Roche Co. The indole compounds were added to the bath fluid dissolved in 0.2–2.0 ml. and neutralized when necessary.

Expression of Results.—The concentrations of the indole compounds are usually represented by their molarity. The inhibitions reported are the average of not less than three measurements. The standard error of the mean has been calculated when necessary.

Manometric Experiments.—The ileum oxygen consumption was measured with Warburg's direct method (see Umbreit, Burris, and Stauffer, 1949) and cholinesterase activity with Ammon's (1933) technique. Rat brain homogenate and blood cells stroma were used for true cholinesterase, and diluted horse serum for pseudo cholinesterase (cf. Todrik, Fellowes, and Rutland, 1951; and Augustinsson, 1950).

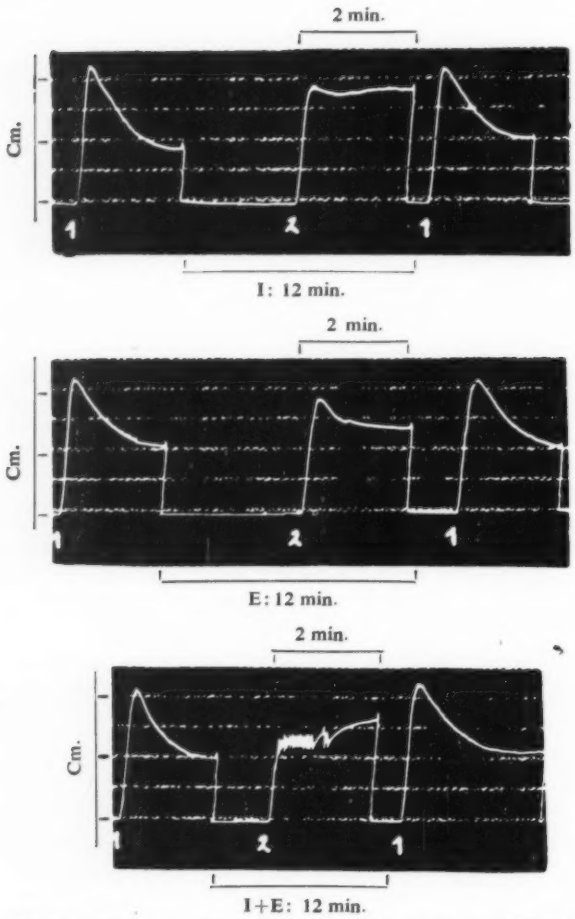

FIG. 1*a*.—Responses of the guinea-pig ileum to 5.0 μg. acetylcholine; I, in the presence of 0.4 mg. indole, and E, in the presence of 0.08 mg. skatole. 20 ml. bath.

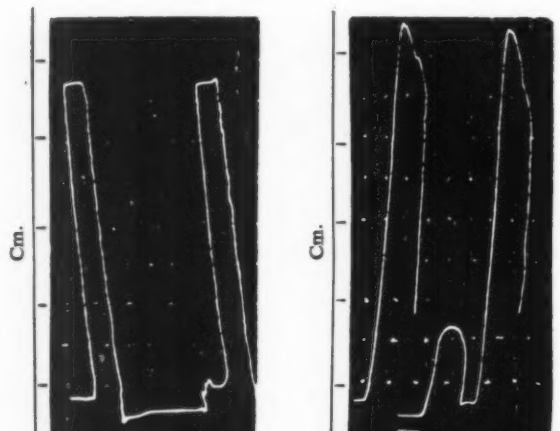

FIG. 1*b*.—Responses of the guinea-pig ileum to 0.1 (I) or 0.4 (E) μg. acetylcholine. I, in the presence of 2.1 mg. indole, and E, in the presence of 0.5 mg. skatole. 20 ml. bath.

RESULTS

Experiments on Guinea-pig Ileum

Stimulant: Acetylcholine.—Skatole and indole strongly affect the intestinal responses to acetylcholine. Both compounds diminish the height of contractions which, however, are better sustained (Fig. 1*a* and *b*). The base line remains unmodified. In the presence of indole, skatole, and acetylcholine, automatic movements of the ileum are frequently observed, which do not occur in the presence of acetylcholine alone. The effects of indole and skatole are additive as shown in Table I.

The inhibitions produced by 9×10^{-4}M indole (with 6.0 μg. acetylcholine as stimulant) after 10 sec., 30 sec., 5, or 10 min. incubation with the organ are respectively 51.5, 70.0, 82.0, and 87.0%. Skatole, 6×10^{-4}M, incubated with the organ for 10 sec., 5, or 10 min., produced inhibitions of 72.0, 94.0, and 93.0% respectively. Therefore, to obtain maximal effects, in all the experiments the organ has been left for 10 min. with the indole compound before adding the stimulant. Indole and skatole inhibitions are reversible, as, after the inhibitor has been removed by washing, the intestine again responds normally to acetylcholine (Fig. 1). However, in some instances recovery takes place at a slower rate and before the normal height is attained smaller contractions may be observed. This also happens with potassium ions as stimulant (Fig. 3).

The effect of several concentrations of indole and skatole on the responses to acetylcholine is shown in Fig. 2. In these experiments, in a preliminary trial a dose of stimulant drug producing a submaximal effect was given repeatedly until the

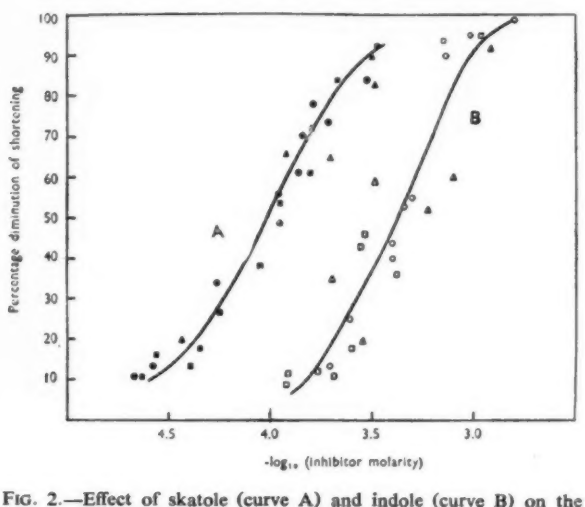

FIG. 2.—Effect of skatole (curve A) and indole (curve B) on the responses of guinea-pig ileum to acetylcholine (●,○), potassium ions (■,□) and histamine (▲,△). Acetylcholine: 5.0–0.2 μg. KCl: 3.0 mg. Histamine: 11.2–1.5 μg. 20 ml. bath.

preparation became stabilized. The doses required to produce these effects ranged from $10^{-6.25}$ to 10^{-8} for acetylcholine and from $10^{-6.15}$ to $10^{-7.73}$ for histamine. For potassium a single dose was used. The effect of inhibitors was then assayed as described under " Methods." The concentration-effect curves obtained are sigmoidal and indicate that relatively small changes in the concentration can strongly modify the extent of the inhibition. The similar shape of the curves for indole and skatole suggests that the mechanism of the inhibition may be the same for both, although the dissociation of the inhibitor-effector complexes are different. The activity of the inhibitor drug has been expressed in terms of pI_{50}, which is the $-\log_{10}$ of the inhibitor molarity reducing by 50% the response to the stimulant. The pI_{50} is 3.4 for indole and 4.0 for skatole, which are equivalent to dilutions of 4.7 and 1.3×10^{-5} respectively. The percentage reduction of the contractions does not seem to be dependent on the concentration or the nature of the stimulant used (Fig. 2).

TABLE I

ADDITION OF THE ACTIONS OF INDOLE AND SKATOLE ON THE GUINEA-PIG ILEUM

Inhibition calculated from experiments as illustrated in Fig. 1. Average response of the ileum to acetylcholine, 2.4 cm. Measurements in triplicate.

Stimulant	Inhibitor	Percentage Diminution of Shortening
Acetylcholine:		
5.0 μg.	1.8×10^{-4}M-indole	12.8±3.6
5.0 μg.	2.7×10^{-5}M-skatole	13.8±3.6
5.0 μg.	Indole and skatole	31.4±2.4
KCl:		
3.0 mg.	1.5×10^{-4}M-indole	10.4±2.2
3.0 mg.	4.2×10^{-5}M-skatole	15.0±0.4
3.0 mg.	Indole and skatole	30.0±1.7

With reference to the prolongation of contraction of the ileum by indole and skatole, it seemed possible that this might be connected with inhibition of cholinesterase, since Waelsch and Rackow (1942) have reported inhibition of serum cholinesterase by indole 10^{-2}M. However, when tested on true or pseudo cholinesterase preparations, indole (2×10^{-3}M) and skatole (6×10^{-4}M) scarcely affected (8 to 12% inhibition) the activity of the enzymes and no explanation for indole or skatole contractures can be provided thereby.

Indican (2×10^{-4}M), indolylacetate (4.3×10^{-4}M), indolylpropionate (4.2×10^{-4}M), and L-tryptophane (2.4×10^{-4}M) do not depress the acetylcholine responses.

Indican and indolylethylamine directly stimulate the ileum (Table II), which confirms earlier observa-

TABLE II

ACTION OF INDICAN AND INDOLYLETHYLAMINE ON ACETYLCHOLINE CONTRACTIONS OF THE GUINEA-PIG ILEUM

Acetylcholine (μg.)	Indole Compound	Height of Contraction Measured from Tracing (cm.)	Percentage Variation
0·3	None	2·8±0·1	—
0·3	2×10^{-4}M-indican	2·9±0·2	+0·3±0·1
2·0	None	3·8±0·1	—
2·0	4×10^{-4}M-indolylethylamine	4·5±0·1	+18·4±0·2

Responses (cm.) to 2.0 10^{-4}M-indican: 1.0±0.1; to 4.0 10^{-4}M-indolylethylamine: 1.1±0.2. The duration of these contractions was short and acetylcholine was added when the organ had returned to the previous relaxed state. All observations were made in triplicate.

tions by Guggenheim and Löffler (1916) with indolylethylamine, and furthermore indolylethylamine slightly potentiates acetylcholine.

Stimulant: Potassium Ions.—Skatole and indole diminish the contractions due to potassium ions and acetylcholine to the same extent, but they cause no prolongation of the contraction such as happens with acetylcholine (Fig. 3). The effect of the two

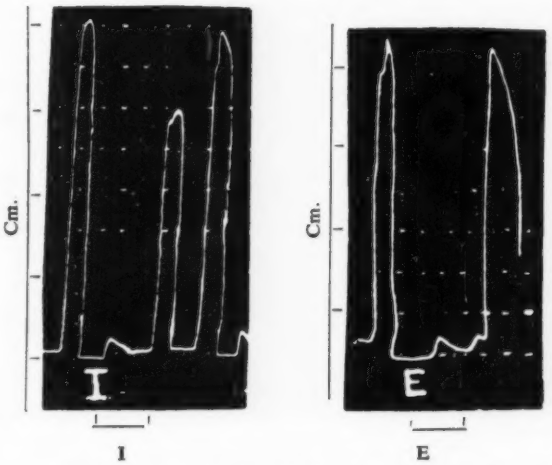

FIG. 3.—Responses of the guinea-pig ileum to 3.0 mg. KCl: I, in the presence of 1.5 mg. indole, and E, in the presence of 0.8 mg. skatole. 20 ml. bath.

compounds on the potassium contractions are additive as shown in Table I. Indican (9×10^{-4}M), indolylacetate (7×10^{-4}M), indolylethylamine (2.2×10^{-4}M), and L-tryptophane (2.4×10^{-4}M) do not affect significantly the potassium contraction.

Stimulant: Histamine.—The ileum responses to histamine (11.2–1.6 μg.) are depressed by indole and skatole in the same way as the potassium contraction. The concentration-effect curve of each compound for histamine is the same as that for potassium and acetylcholine (Fig. 2).

Effect of Indole and Skatole on the Oxygen Consumption of the Intestine

Intestinal contractility depends on the oxygen supply to the organ (Furchgott and Shorr, 1950), and as indole inhibits cell respiration (Quastel and Wheatley, 1933) it seemed of interest to find out whether there was some relationship between inhibition of contraction and depression of respiration of the intestine. Small pieces of guinea-pig ileum (26.0–41.0 mg., dry weight) were therefore suspended in Krebs-Ringer-phosphate solution and their oxygen uptake measured in Warburg manometers. The oxygen uptake (Qo_2: −4.1) did not change significantly after 15 min. incubation with concentrations of indole (1.7×10^{-3}M) or skatole (2×10^{-4}M) which strongly diminish the contractility.

Experiments on Other Tissues

Large Intestine.—Guinea-pig, mouse, or rat colon was used. The colon wall is thicker than the ileum, especially in the rat and the guinea-pig, which might affect the diffusion of stimulants or inhibitors to the muscle fibre. Nevertheless, indole and skatole depress the contractility, their action being more marked on the descending colon. Furthermore, they also diminish the gut tonus (Fig. 4),

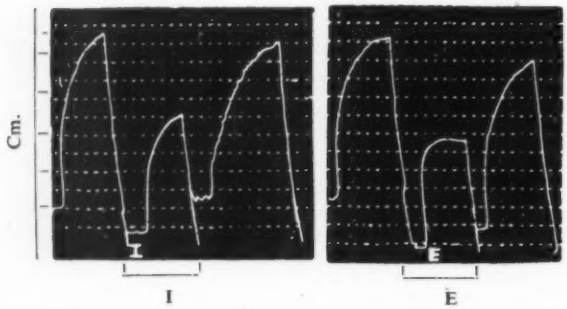

FIG. 4.—Responses of the guinea-pig colon to 3.5 μg. acetylcholine; I, in the presence of 1.8 mg. indole, and E, in the presence of 2.5 mg. skatole. 20 ml. bath.

and when acting together there is summation of effects (Table III). Skatole is somewhat less effective on the colon than on the ileum, whereas indole depresses the responses of both organs to the same extent.

Rabbit Duodenum.—Indole and skatole diminish the height of the rabbit duodenum contractions, but do not affect their frequency (Fig. 5).

Rat Uterus.—Indole and skatole diminish both the height and frequency of contractions of the rat uterus (Fig. 6), and its responses to the pituitary oxytocic hormone. Indole (6×10^{-4}M) and skatole (4×10^{-4}M) depress by 54 and 34%, respectively, the contractions elicited by 0.1 i.u. oxytocic hormone (a " Pitocin " preparation).

TABLE III

ACTION OF INDOLE AND SKATOLE ON ACETYLCHOLINE CONTRACTIONS OF THE COLON

Average response to the stimulant (measured from the tracing) of the rat colon, 5.0 cm ; the mouse colon (ascending), 3.6 cm ; and mouse colon (descending), 5.0 cm Observations were made in triplicate.

Organ	Inhibitor	Depression of Tonus Measured from the Tracing (cm.)	Acetylcholine (μg.)	Percentage Diminution of Shortening*
Rat colon (ascending)	8×10^{-4}M-indole	0·5±0·1	0·7	69·3±0·3
,, ,,	4×10^{-4}M-indole	0·0	0·7	36·7±1·7
,, ,,	2×10^{-4}M-skatole	0·2±0·0	0·7	21·2±3·0
Mouse colon (ascending)	8×10^{-4}M-indole	0·0	15·0	30·6±3·8
,, ,,	2×10^{-4}M-skatole	0·0	15·0	17·7±0·8
,, ,,	Indole and skatole	0·0	15·0	69·5±1·5
Mouse colon (descending)	8×10^{-4}M-indole (*a*)	0·7±0·0	3·0	79·0±3·6
,, ,,	4×10^{-4}M-indole (*b*)	0·0	3·0	33·5±1·5
,, ,,	2×10^{-4}M-skatole	0·0	3·0	38·2±2·2
,, ,,	Indole (*b*) and skatole	1·1±0·0	3·0	61·0±3·2

* Change of tonus accounted for.

Striated Muscle.—When tested on the *Leptodactylus ocellatus* (L.) Gir. anterior rectus abdominis, indole potentiates acetylcholine, while indole, skatole, indican, and indolylpropionate potentiate the effect of potassium ions (Table IV). Indolylacetate (5.9×10^{-4}M), indolylethylamine (2.2×10^{-4}M), and L-tryptophane (2.4×10^{-4}M) do not affect the responses to acetylcholine or potassium.

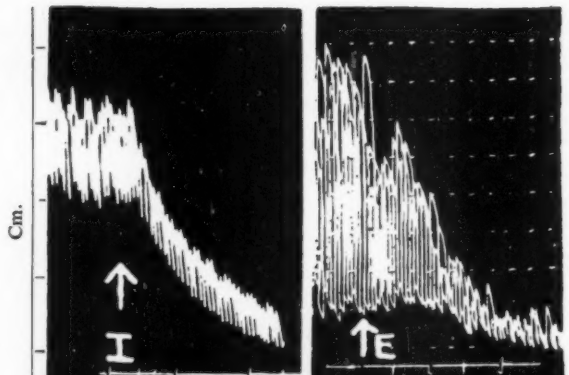

FIG. 5.—Effect of indole on contractions of the rabbit duodenum. I, addition of 1.1 mg. indole. E, addition of 2.5 mg. skatole. Time marks, 30 sec. 20 ml. bath.

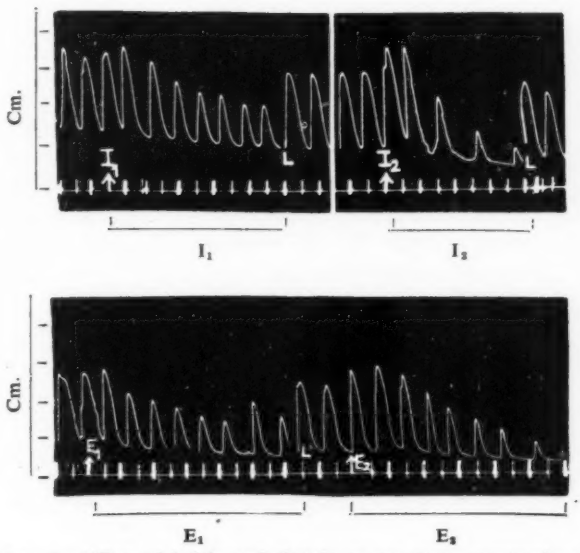

FIG. 6.—Effect of indole and skatole on rat uterus contractions. I_1, addition of 1.3 mg. indole; I_2, 2.6 mg. indole; E_1, 0.5 mg. skatole; E_2, 1.0 mg. skatole. Time marks, 30 sec. 20 ml. bath.

TABLE IV

ACTION OF INDOLE AND SOME INDOLE COMPOUNDS ON THE CONTRACTIONS OF THE FROG RECTUS ABDOMINIS

Stimulant	Indole Compound	Height of Contractions Measured from Tracing (cm.)	Percentage Variation
Acetylcholine:			
2·0 μg. ..	None	2·8±0·0	—
2·0 ,, ..	$1·1\times10^{-4}$M-indole	3·8±0·2	+35·6±0·1
20·0 ,, ..	None	2·3±0·0	—
20·0 ,, ..	4×10^{-5}M-skatole	2·3±0·0	0·0±0·0
KCl:			
3·0 mg. ..	None	2·6±0·1	—
3·0 ,, ..	$8·5\times10^{-4}$M-indole	2·9±0·1	+10·3±0·06
3·0 ,, ..	None	3·4±0·1	—
3·0 ,, ..	6×10^{-5}M-skatole	4·4±0·1	+29·4±0·05
3·0 ,, ..	None	1·5±0·1	—
3·0 ,, ..	9×10^{-4}M-indican	2·0±0·1	+33·1±0·11
3·0 ,, ..	None	1·7±0·1	—
3·0 ,, ..	7×10^{-4}M-indolyl-propionate	2·0±0·1	+17·6±0·10

Observations were made in triplicate.

DISCUSSION

It seems to be the rule that the contractility of smooth muscle organs (as observed with duodenum, ileum, colon, and uterus) is depressed by indole and skatole. It has been shown by Feldberg (1951) that histamine acts on the muscle fibre whereas potassium and acetylcholine act both on the muscle fibre and the myenteric plexuses. Since the action of these three drugs is equally depressed by indole and skatole, it seems likely that the latter act on the final common path of the three drugs, namely the smooth muscle fibre itself. This is confirmed by the finding that indole and skatole also inhibit the action of the pituitary oxytocic hormone on the uterus (see Stehle, 1950).

The guinea-pig ileum preparation is normally quiescent *in vitro*, probably owing to the fact that little acetylcholine is released under these conditions. When the intestine is *in situ* a continuous release of acetylcholine occurs, as shown by Feldberg and Rosenfeld (1933), and on this basis it is reasonable to compare the reactions of the tissues *in situ* to those of the isolated organs in the presence of acetylcholine. Our experiments may thus furnish some explanation of the contradictory results by other workers with indole and skatole in the whole animal. Thus, Ets and Feinberg (1942) obtained paralysis of the dog jejuno-ileum by injecting intravenously 25 or 60 mg. indole per kg., which if diluted evenly in the body water (cf. Fulton, 1946) would give a concentration of 3 to 8×10^{-4}M. These concentrations have been shown in our experiments to depress the smooth muscle response to acetylcholine, potassium ions, and histamine. On the other hand, the more sustained contraction of the ileum by acetylcholine, in the presence of indole and skatole, may explain the apparent stimulation of intestinal motility observed with these substances by Biebl (1929) and Ets and Feinberg (1942). *In vitro*, the inhibitions of spontaneous intestine motility observed by Danilewsky (1908a and b), Guggenheim and Löffler (1916), Waddell (1927), and Garcia Blanco *et al.* (1941) were obtained with 8×10^{-4} to 8.6×10^{-2}M-indole or 3.8×10^{-4}M-skatole (concentrations recalculated from the experimental data reported in the papers quoted), in good agreement with our own results.

With striated muscle preparations, our results, like Torda and Wolff's, contrast with those of Danilewsky (1908a and b), Waddell (1927), and Yanai (1935), who obtained depression of the contractility of the frog heart or gastrocnemius by skatole or indole, although it must be pointed out that below a concentration of 0.01% indole may stimulate the frog heart (Danilewsky, 1908b). The different behaviour of the amphibian rectus abdominis fits in with other structural and functional peculiarities of the muscle, described already by Kuffler (1949).

Among the several compounds studied, the least soluble in water are the most effective on muscle. It is evident that the presence of polar groups in the molecule increases the solubility and diminishes the pharmacologic action; in some instances, as with indican, the effect is even reversed. The detoxication of indole in the body by changing it into indican nullifies its ability to inhibit smooth muscle contraction. On the other hand, the

presence of an amino group in the side chain confers on the molecule the property of stimulating the intestine, and in this connection it is of interest that 5-hydroxytryptamine (enteramine) has been claimed to be the hormone of the intestinal enterochromaffine tissue (Erspamer and Asero, 1952).

Indole and skatole seem to be normal constituents of the intestinal fluids, and, as reported by Herter (1907), there may be up to 60 mg. indole and 10 mg. skatole in 100 g. faeces. The concentrations calculated from these figures tally with those found in our experiments to affect intestinal motility. It may therefore be considered probable that these substances can play a role in intestinal pathology, especially when the accessibility to the muscle layer of the gut is increased by lesions of the mucosae (Nicolai, 1941a and b, and 1942).

Summary

1. The effects of indole, skatole, indoxylsulphate, β-indolylethylamine, indolylacetate, β-indolylpropionate, and L-tryptophane on the responses of striated and unstriated muscles to their chemical physiological stimulants have been studied.

2. Indole and skatole diluted about 1 in 10^5 diminish the shortening of guinea-pig ileum by acetylcholine, potassium ions, and histamine, and also prolong the contraction of the ileum due to acetylcholine. The effect of these inhibitors are additive and cannot be attributed to block of the oxygen consumption of the intestine or to inhibition of cholinesterase activity.

3. Indolylethylamine slightly potentiates the effect of acetylcholine, leaving unaffected the response to potassium ions. Indoxylsulphate, indolylacetate, indolylpropionate, and L-tryptophane do not modify the response of the ileum to acetylcholine and potassium ions.

4. Indolylethylamine and indoxylsulphate cause a contraction of the guinea-pig ileum.

5. Indole and skatole diminish the tonus of the colon of the guinea-pig, rat, and mouse, and also diminish the responses to acetylcholine and potassium ions; their effect is stronger on the terminal part of the colon.

6. Indole and skatole diminish the automatic contractions of the duodenum of the rabbit and of the uterus of the rat, and also the responses of the latter to the pituitary oxytocic hormone.

7. Indole potentiates acetylcholine effects, and indole, skatole, indoxylsulphate, and indolylpropionate potentiate potassium effects, on the frog anterior rectus abdominis.

8. It is considered that the depressant effects of indole compounds are due to a direct action on the smooth muscle fibres.

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THE AFFERENT PATHWAY OF THE BEZOLD REFLEX: THE LEFT VAGAL BRANCHES IN DOGS

BY

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(RECEIVED JUNE 23, 1953)

In experiments on cats Jarisch and Richter (1939) and Jones (1953) had difficulty in locating the afferent pathway for the Bezold reflex. Even on the right side, which was the simpler, the distribution was not easy to follow. Several difficulties were encountered in these experiments, among which was the doubt that existed in any given cat as to whether a particular dose of the veratrum alkaloids was causing a fall of blood pressure and heart rate by the Bezold reflex or by a central action. In the dog it is comparatively easy to cannulate the main branches of the left coronary artery, and hence to localize the site of action more certainly.

This paper describes experiments which have identified the afferent pathway on the left side in dogs, and also describes the nature of some afferent fibres from these nerves, but probably not those responsible for the Bezold reflex.

METHODS

Forty-six dogs were used in these experiments. They were anaesthetized with sodium pentobarbitone 32 mg./kg. administered intravenously. Artificial ventilation was applied through a tracheal cannula; the chest was opened down the midline and the incision extended between the third and fourth ribs on the left side to allow adequate access to the heart. The blood pressure was recorded from a femoral artery either by a mercury manometer or by a condenser manometer having a frequency response of 200 cycles/second or better.

The peripheral end of the left circumflex coronary artery was cannulated at its origin (see insert to Fig. 2) and connected by a short length of flexible rubber tubing to the central end of the right internal mammary artery, so as to facilitate repeated injections of not more than 0.1 ml. veratridine hydrochloride dissolved in physiological saline. The dog was heparinized.

Small nerve-slips were dissected off branches of the vagus after they had left the main trunk, and action potentials were recorded from these, using a resistance-capacity amplifier, and were displayed on a cathode-ray oscilloscope. The amplifier had a maximum discrimination factor of 1:50,000, an equivalent noise resistance of 6,000 ohms, a high pass filter (cut off 75 c/s), and a low pass filter (cut off 5,000 c/s). One of the principal difficulties in recording action potentials from small fibres so near the heart is the relative size of the electrocardiogram, which is also picked up. In most experiments this source of interference was reduced to a reasonable level by using this type of amplifier, which has an exceptionally high discrimination for out-of-phase signals, and by earthing the tissues around the insulated dissecting platform. In some later experiments a polished stainless steel dissecting platform was earthed, and a single electrode used for recording the action potentials. The electrocardiogram was recorded from leads 1 or 2.

RESULTS

The Afferent Nerve Path of the Bezold Reflex

Preliminary observations showed that there were three problems to be overcome if experimental results were to be of value.

1. It is known (Krayer, Wood, and Montes, 1943) that veratridine injected into dogs causes a fall of blood pressure and heart rate, both by an action on receptors in the coronary vessels (the Bezold reflex) and by a central action. Consequently, the intravenous injection of veratridine in some dogs leads to a fall of blood pressure which is not entirely abolished by cutting the vagi. Therefore, after a few preliminary experiments in which the central effect of veratridine was encountered, we restricted our observations to dogs in which veratridine was administered only into the left circumflex coronary artery. The dose required to cause a fall of blood pressure on intracoronary arterial injections is about 1 μg., one-tenth to one-twentieth that required on intravenous injection.

2. The fall of blood pressure on injection of veratridine is due not only to peripheral vasodilatation (Krayer, Moe, and Mendez, 1944; Moe, Bassett, and Krayer, 1944; Dawes, Mott, and Wid-

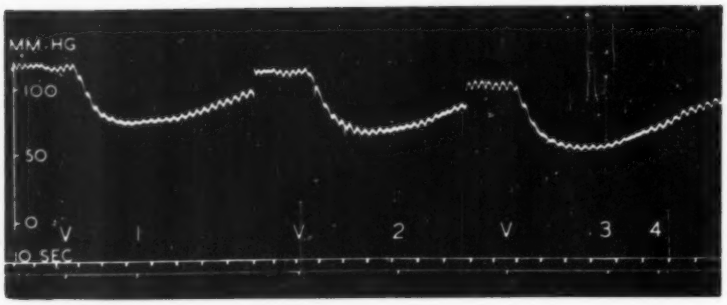

FIG. 1.—Dog, 11 kg.; pentobarbitone anaesthesia. R. vagus cut. Record of femoral arterial pressure with a mercury manometer. Injection of 1 μg. veratridine (at V) into the left circumflex coronary artery at 10 min. intervals caused a fall of blood pressure, which was not affected by section of the main left vagal trunk below the aortic arch (at 1), of the left anterior cardiac branches (at 2) or left depressor branches (3 and 4).

dicombe, 1951), but also to cardiac slowing. Hence, if section of a vagal branch leads to a reduction in the fall of blood pressure observed after administration of veratridine, it might be objected that this would result from interference with efferent nerves to the heart. However, we have repeatedly observed that, in dogs in which the *right* vagus is cut, injection of veratridine into the left circumflex coronary artery causes a fall of blood pressure without any significant change in heart rate (Fig. 3). In one experiment, for instance, the heart rate and P–R interval (measured from the electrocardiogram) changed by less than 5%, which was within the limits of accuracy of the measurements.

Although, therefore, we share the widespread impression that the afferent fibres of the Bezold reflex in the dog travel up both vagus nerves, we have confined our observations to the left vagus. The right vagus was cut early in the course of every experiment.

3. The injection of repeated doses of the veratrum alkaloids leads rapidly to decreasing responses. Veratridine has been used for these experiments because it is less liable to cause tachyphylaxis than other veratrum alkaloids. The doses administered have been as small as possible (about 1 μg.) consistent with obtaining an adequate fall of blood pressure (30–50 mm. Hg). Experience showed that a time interval of ten minutes between doses was a reasonable compromise between the dangers of tachyphylaxis and deterioration of the preparation. Even so, tachyphylaxis was observed occasionally; such experiments have been excluded from further consideration.

In the first eight successful experiments the main thoracic branches of the left vagus were cut in succession (the right had of course been cut at the beginning of the experiment). The procedure adopted was not exactly the same in all experiments, deliberately, since the risk of encountering tachyphylaxis progressively increased with time. A good experiment is illustrated in Fig. 1, in which it is seen that section of the main left vagal trunk below the arch of the aorta (after it had given off its cardiac branches but before reaching the root of the lungs) and section of the left depressor branches from the aorta (running medially towards the origin of the brachiocephalic artery, Fig. 2) did not reduce the size of the Bezold reflex. In five dogs there was conclusive evidence that the main afferent pathway of the Bezold reflex did not run in the main left vagal trunk below the arch of the aorta, in four dogs the aortic depressor nerves were excluded, and in six dogs the anterior cardiac nerves, which run across the pulmonary artery to the left atrium and ventricle, were also

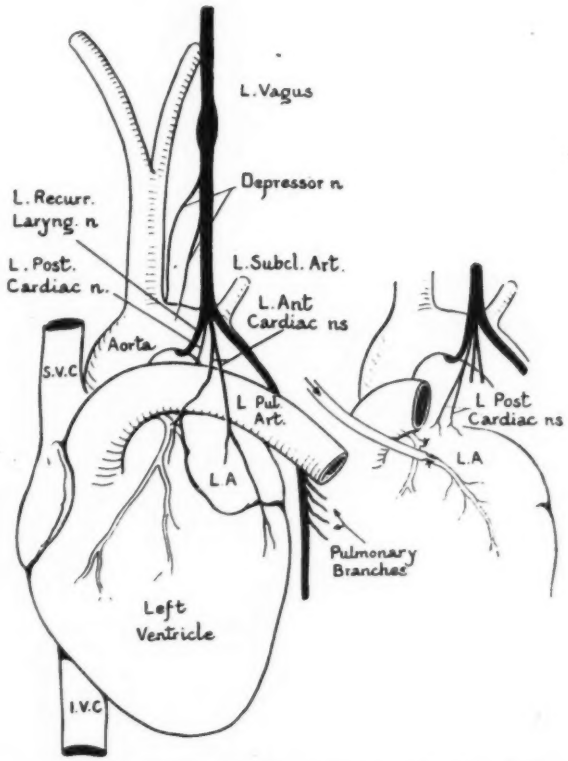

FIG. 2.—Anterior view of the heart to show the distribution of the principal branches of the left vagus. The insert shows the disposition of the left posterior cardiac nerves in relation to the left pulmonary artery and left atrium (L.A.); the left circumflex coronary artery has been cannulated.

excluded. In two dogs the observations suggested that the afferent pathway lay among vagal branches which dived down between the aortic arch and the left pulmonary artery (Fig. 2).

In the next series of five successful experiments attention was concentrated on these posterior cardiac branches of the vagus. Section of this group of nerves, sometimes including the left recurrent laryngeal, at the point where they cross the aortic arch, abolished the Bezold reflex. But it was thought that the afferent nerve fibres only joined the left recurrent laryngeal at the level of the aortic arch, since in two of the five dogs the section did not include the latter.

In order to establish as far as possible the origin of the afferent nerve fibres, a more extensive dissection was undertaken. The left pulmonary artery was tied and divided, together with all vagal branches running anteriorly to it, and the main vagal trunk below the aortic arch (see insert to Fig. 2). This exposed the inferior aspect of the aortic arch, with the left recurrent laryngeal, cardiac, and other branches of the vagus running round it. A few (usually two) very small nerves were seen running to the aortic arch from the base of the left atrium. In all five dogs in which this preparation was completed the Bezold reflex was present, in spite of the extensive dissection and the section of main vagal trunk described. In all five, cutting the fine nerves running from the left atrium abolished the reflex (Fig. 3).

Action Potentials

Twenty-one single and multifibre preparations were made from the aortic depressor branches of the vagus. Intravenous injections of 30–40 μg. veratridine did not sensitize any of these endings.

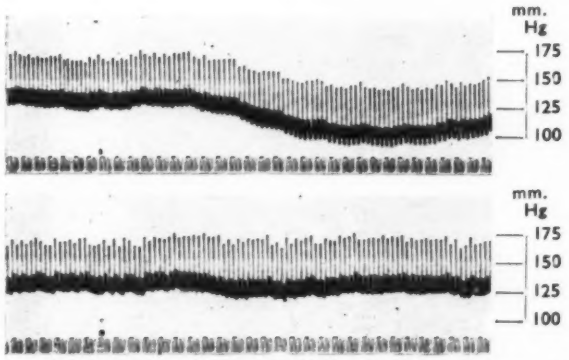

FIG. 3.—Dog, 9 kg.; pentobarbitone anaesthesia. R. vagus cut; left pulmonary artery divided and retracted to gain access to the nerves running beneath it. Record of femoral arterial pressure with a condenser manometer; time signals in 1/10 seconds, interrupted once per second. At the signal marks 1 μg. veratridine was injected into the left circumflex coronary artery. Between the upper and lower records there was a 10 minute interval during which the left posterior cardiac branches of the vagus were divided.

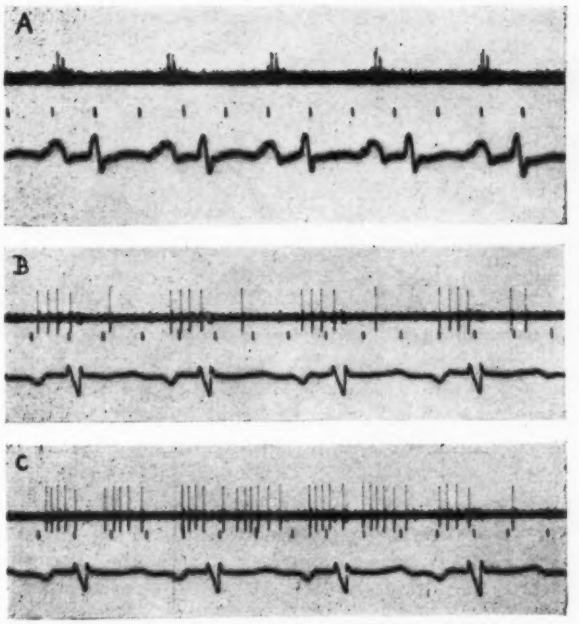

FIG. 4.—Records of action potentials recorded from the left posterior cardiac nerves (above), time marks at 1/10 sec. intervals, and the electrocardiogram (below). The upper record (A) shows a fibre which discharged only between the P and QRS deflections of the E.C.G. The lower record (B and C) shows a fibre which discharged both before and after the QRS complex, the discharge frequency varying with different phases of the respiration.

From the small cardiac branches of the vagus running behind the left pulmonary artery from the back of the heart, 14 single and multifibre preparations were made. The spontaneous activity recorded in the majority of these preparations was similar to that described for atrial pressure or volume receptors by other workers (Paintal, 1953). Some discharged only immediately after the P wave of the electrocardiogram (Fig. 4, A), others discharged both between the P and QRS waves and again about the T wave (Fig. 4, B and C). A few only discharged at or after the T wave. These fibres discharged more frequently on intravenous injection of saline, but were not sensitized by the injection of 30–40 μg. veratridine intravenously, or 1–2 μg. veratridine into the left circumflex coronary artery. In all these preparations both vagi were cut in order to exclude reflex changes in the circulation. In three preparations, however, injection of veratridine into the left circumflex coronary artery caused an audible and visible increase in background activity ; the characteristic spontaneous discharge of the fibres responsible for this phenomenon could not be identified. We had the impression, since we were at the time recording from very small nerve strands, that these nerve fibres must have been small. In addition, on several occasions we encountered single fibre

preparations from these nerves which showed spontaneous activity with cardiac and/or respiratory rhythms which we were unable to classify with known types of receptor.

Discussion

We have confined our attention to the vagal branches of the left side of the heart in dogs because preliminary experiments showed that, when the right vagus was cut, injection of veratridine into the left coronary arteries no longer caused any significant bradycardia. Thus, with the right vagus cut, the Bezold reflex is presumably due almost entirely to peripheral vasodilatation. Section of branches of the left vagus then causes a reduction in the Bezold reflex only by interruption of afferent nerve fibres, and not by interruption of efferent vagal cardio-inhibitory nerve fibres. The delineation of the afferent pathway on the left side is therefore a simpler proposition than that on the right side.

Progressive section of the left vagal branches showed that the afferent nerve fibres for the Bezold reflex accompany the left recurrent laryngeal nerve as it runs across the aortic arch, and then run posterior to the left pulmonary artery towards the back of the left atrium. Comparison of the distribution of these branches with the anatomical studies of Schurawlew (1928) shows no exact correspondence with those which he described; the nearest seem to be the "rami cardiaci sin_3 zum 4 Geflecht" ($r.r.c.s_3$ of his Fig. 1). We propose to refer to these nerves as the left posterior atrial branches of the vagus. They are thus distinguished from the vagal branches to the heart which run anterior to the left pulmonary artery; the term atrial should not be taken to imply that the nerve fibres end in the atrium only.

It was disappointing to find that the only fibres encountered in any number, which almost certainly originate from atrial pressure or volume receptors, were not sensitized by veratridine. While there is good reason to believe that there are other types of fibres running among these nerves, it is not surprising that under the particular experimental conditions smaller nerve fibres were not isolated. In so far as these observations can be compared with those of Jarisch and Zottermann (1948) on the right atrial vagal nerve fibres of the cat, they would agree in the general conclusion that the Bezold afferent nerve fibres are probably of small diameter and accompany the vagal branches to the atria.

Summary

The afferent pathway for the Bezold reflex has been defined on the left side in dogs by injecting veratridine directly into the left circumflex coronary artery; the right vagus was cut. The afferent nerves run from the vicinity of the left atrium beneath the left pulmonary artery to join the left recurrent laryngeal nerve as it crosses the aortic arch.

One of us (J. G. W.) is indebted to the Medical Research Council for a grant during this work. We are most grateful to Professor O. Krayer for the sample of veratridine.

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HAYATIN METHIODIDE: A NEW CURARIFORM DRUG

BY

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(RECEIVED JUNE 15, 1953)

While investigating the pharmacological properties of an alkaloid "hayatin" from the root of an indigenous plant, *Cissampelos pareira*, Linn., its methiodide* was found to depress respiratory movements and, on further investigation, to be a muscle relaxant. In a preliminary note (Pradhan, Ray, and Varadan, 1952), the curariform property of this compound was briefly reported. It is a colourless, crystalline substance, m.p. 281° C. (decomp.), soluble in warm water and less so in cold water. Its empirical formula is $C_{35}H_{36}O_6N.CH_3I$† (Bhattacharya, Sharma, and Dhar, 1952). In the present paper the curariform activities of hayatin methiodide and of *d*-tubocurarine chloride are compared.

MATERIAL AND METHODS

Several species of animals were studied. A 1% solution of crystalline hayatin methiodide in distilled water and a 1% solution of *d*-tubocurarine chloride (Burroughs Wellcome and Co.) were used throughout unless otherwise stated.

Mice.—Thompson's inclined-screen procedure for bioassay of insulin was adapted for the quantitative evaluation of curariform activity in mice (Thompson, 1946). Hoppe (1950) compared the curariform activity of Win 2747 and *d*-tubocurarine chloride by this method. In the present study, doses of hayatin methiodide, at intervals of 0.05 mg./kg. in a volume of 0.01 ml./g. body weight, were injected subcutaneously to groups of 5 mice. The mice were placed in small stalls, 6 in. × 12 in., on a fine-mesh wire screen inclined at an angle of 50° to the horizontal plane. The dose producing typical skeletal-muscle paralysis in a mouse, and causing it to slide down the screen abruptly within half an hour after injection, was considered to be the effective dose for that animal. The percentage of mice developing paralysis at each dose level was determined. The effective dose (ED50) and its standard error were calculated by Kärber's method (Finney, 1952).

Rabbits.—Curariform activity of hayatin methiodide was investigated in rabbits by a quantal assay procedure (Hoppe, 1950). Suitably graded doses of the substance, in a volume of 1.0 ml./kg., were given intravenously, at the rate of 1.0 ml./5 sec., to groups of 10 rabbits. After injection the rabbits were placed in a large enclosure on the floor for detection of head-drop. The response was considered positive when the head dropped forward on the supporting surface and could not be raised in spite of a light tap on the snout of the animal. The percentage of rabbits showing head-drop at each dose level was determined. The head-drop dose (HD50) and the standard error were calculated by Kärber's method. Mean head-drop was determined for hayatin methiodide by using concentrations of 0.025 mg./ml./min. Comparison of *d*-tubocurarine chloride and hayatin methiodide was made by both a quantal assay procedure and a cross-over test.

Cats and Dogs.—Sciatic-gastrocnemius preparations were prepared in cats and dogs anaesthetized with quinalbarbitone or sodium phenobarbitone as described by Burn (1950). A piece of the bone into which tendo Achilles is inserted was cut through, and a stout cord tied round the tendon was passed over a pulley to a suitable weight. A light thread, also fastened to the tendon, was connected to a writing lever for recording the individual muscle contractions. The sciatic nerve was dissected, crushed at the proximal end, and stimulated by means of an electronic stimulator giving impulses of 0.001 sec. duration at the rate of 15 per min. After a control period of repeated stimulation, during which a series of contractions of uniform height was recorded, hayatin methiodide was injected intravenously in a suitable dose. When the muscle contraction and respiration had returned to normal, a further dose of the same drug, or of *d*-tubocurarine chloride, was injected. Percentage paralysis in the muscle was calculated from the diminution in the height of contraction from its original level. Muscles were stimulated directly by pulses of 0.005 sec. duration at the rate of 15 per min. Carotid blood pressure and tracheal air pressure were also recorded in these animals, and arrangements were made for artificial respiration when necessary. Diaphragmatic respiration was recorded directly by means of a light thread fixed to the peritoneal aspect of the right hemidiaphragm. Intercostal movement was recorded by a thread tied round the lateral part of the 6th, 7th, or 8th rib.

Frog Rectus Abdominis.—The rectus abdominis muscle was dissected from a pithed frog and was put for one hour in Ringer solution containing 1 in

* Isolated and prepared in the Medicinal Chemistry Division of the Central Drug Research Institute, Lucknow.

† Now modified to $C_{35}H_{36}O_6N.2CH_3I$.

,500,000 eserine. It was then put in a 50-ml. bath already filled with eserinized Ringer solution. After four minutes this fluid was replaced by ordinary frog-Ringer solution. A dose of hayatin methiodide was added and the effect noted. A buffered solution of acetylcholine (0.005%) was then added to the same bath, 1 ml. every two minutes till the muscle contracted. The muscle was washed with ordinary frog-Ringer solution and the effect of acetylcholine alone was tested on the same muscle. Another dose of hayatin methiodide was then tried, either on the same or on a different muscle.

Toxicity was studied in mice (18–22 g.), rabbits (about 2 kg.), and dogs.

Results

Curariform Activity

Mouse Inclined-screen Method. — Hayatin methiodide, injected subcutaneously into mice, produced restlessness within two to five minutes; the animals gradually became quiet and signs of respiratory embarrassment became apparent. Paralysis of skeletal muscles started in the hind-leg and increased till the mice lost their ability to retain their position on the wire mesh of the screen and abruptly slid down. With increasing doses the interval between the injection of the drug and the onset of paralysis diminished and the duration of paralysis of limb muscles increased. Table I shows the effect of various doses on the onset and duration of paralysis. After a while the animals were able to get up slowly from the lying down position, and after longer intervals they were able to move about with complete recovery from paralysis. Death from high doses, and also from low doses in susceptible individuals, took place from respiratory failure. In such animals twitchings of the limbs, and micturition, occurred.

TABLE I

ONSET AND DURATION OF PARALYSIS AFTER SUBCUTANEOUS INJECTION OF HAYATIN METHIODIDE TO MICE

Dose (mg./kg.)	No. of Animals Used	No. of Animals Paralysed	Interval between Injection and Onset of Paralysis (min.)	Duration of Paralysis (min.)
0·15	5	0	—	0
0·20	5	3	9±2·18	13±11·42
0·25	5	5	9±2·40	21± 5·47
0·30	5	5(a)	6±1·41	39± 4·84
0·35	5	5(b)	6±2·03	30± 9·70
0·40	5	5(c)	3±0·90	

(a) One died after 43 min.
(b) ,, ,, ,, 14 ,,
(c) All died with paralysis after 23±7·8 min.

The ED50 in mice, after subcutaneous injection, was 0.195±0.0187 mg./kg., which is about half the ED50 for *d*-tubocurarine chloride obtained by Hoppe.

Rabbit Head-drop Method.—The head-drop potency (HD50) in rabbits by a quantal assay procedure was 0.0526±0.0009 mg./kg. for hayatin methiodide, and 0.1124±0.0113 mg./kg. for *d*-tubocurarine chloride. Hayatin methiodide thus appeared to be 2.13 times as potent as *d*-tubocurarine chloride (Table VI). The HD50 for *d*-tubocurarine chloride obtained by this procedure agrees with the value of between 0.110 to 0.115 mg./kg. obtained by Collier, Paris, and Woolf (1948). Everett (1948) and Hoppe (1950), however, gave higher values for the HD50—of 0.15 mg./kg. and 0.146 mg./kg. respectively.

The ratio of HD50 to LD50 was 1.34 for hayatin methiodide, as compared with 1.28 for *d*-tubocurarine chloride. After rapid intravenous injection of hayatin methiodide, symptoms similar to those produced by *d*-tubocurarine chloride appeared. At first there was paralysis of the hind legs followed by respiratory depression and marked skeletal muscle incoordination, then slight restlessness; head-drop finally occurred within 1 to 2 minutes. Data showing the duration of head-drop and limb muscle paralysis with different doses of hayatin methiodide are given in Table II. It is evident that the duration of paralysis of neck and limb muscles was prolonged with increase of dose.

TABLE II

DURATION OF HEAD-DROP, AND TIME FOR COMPLETE RECOVERY FROM LIMB PARALYSIS, AFTER INTRAVENOUS ADMINISTRATION OF VARIOUS DOSES OF HAYATIN METHIODIDE TO RABBITS

Dose (mg./kg.)	No. of Animals Used	Duration of Head-drop (min.)	Duration of Limb Paralysis (min.)
0·052	10	1·8±1·88	2·3±2·12
0·054	10	2·0±1·82	4·5±2·31
0·057	10	2·1±1·39	5·0±2·26
0·065	10 (a)	4·0±3·34	6·34±3·22
0·070	10 (b)	6·96±2·85	8·67±3·39
0·075	10 (c)	9·04±4·16	11·19±4·16

(a) 3 died after 8·25±1·95 min.
(b) 6 ,, ,, 7·18±2·06 ,,
(c) 6 ,, ,, 10·15±1·61 ,,

The potencies of these two drugs were also compared in rabbits by a cross-over test. By this method the mean head-drop dose was 0.0840±0.0176 mg./kg. for hayatin methiodide and 0.1713±0.0364 mg./kg. for *d*-tubocurarine chloride. The standard error of the difference was 0.0283, with "t"=0.79; the difference is thus significant. By this method hayatin methiodide was found to be 2.04 times as active as *d*-tubocurarine chloride. The mean head-drop dose for

hayatin methiodide was found to be much lower than the figure obtained previously (Pradhan *et al.*, 1952), probably because a purer sample of the methiodide was available. Our figure for *d*-tubocurarine chloride appears to be a little higher than that reported by other workers.

The " cumulative " effect of hayatin methiodide, as manifested by the variation in response of the head-drop when the drug was given on successive days, was also studied. Three groups of five rabbits were each given a different dose of the drug (0.050, 0.052, and 0.055 mg./kg.), by rapid intravenous injection, daily for five successive days. The results are given in Table III. It appears from the data that there was no consistent alteration in sensitivity with repeated dosing.

TABLE III

DAY-TO-DAY VARIATION IN HEAD-DROP RESPONSE OF RABBITS TO HAYATIN METHIODIDE GIVEN INTRAVENOUSLY ON 5 SUCCESSIVE DAYS

Dose (mg./kg.)	Rabbit No.	Head-drop Responses. Day: 1	2	3	4	5	Total No. Head-drops/ Total No. Injections
0·050	37	−	−	−	−	−	10/25
	38	−	+	−	+	+	
	39	−	+	+	+	−	
	41	+	−	−	+	+	
	51	−	+	−	−	−	
0·052	42	−	−	+	+	+	12/25
	43	−	+	−	+	+	
	44	+	+?	−	+	+	
	45	+	+	−	−	−	
	47	−	−	−	−	−	
0·055	16	+	+	+	+	+	23/25
	22	+	+	+	+	+	
	53	−	+?	+	+	+*	
	55	+	−	+	+	+	
	56	+	+	+	+	+	

* Died afterwards.

Cat and Dog Nerve-muscle Preparations.— Though there was considerable individual variation in the relation between the dose of hayatin methiodide and the percentage paralysis of the gastrocnemius, the dose-response curve appeared to be linear ($Y = 37.22 + 136.76\ X$) over the range of doses used. The average time taken for complete recovery of the nerve-muscle preparation was approximately an hour in each instance. There was no evidence to indicate an antagonism between *d*-tubocurarine chloride and hayatin methiodide when both were injected in the same animal at suitable intervals ; on the other hand a slight synergistic effect was sometimes manifested.

A comparison of hayatin methiodide and *d*-tubocurarine chloride was carried out, both the drugs being administered to the same animal at suitable intervals. Table IV shows the results.

TABLE IV

COMPARISON OF PARALYSING POTENCY OF HAYATIN METHIODIDE AND *d*-TUBOCURARINE CHLORIDE ADMINISTERED INTRAVENOUSLY TO DOGS

Dog No.	*d*-Tubocurarine Chloride (mg./kg.)	Hayatin Meth. (mg./kg.)	Max. Twitch Depression (% of Control)	Ratio— Hayatin Methiodide/ *d*-Tubocurarine Chloride
56	0·29	—	75	1·01
	—	0·29	76	
57	0·26	—	49	1·35
	—	0·26	66	
58	0·33	—	93	0·97
	—	0·33	90	
146	—	0·10	41	1·22
	0·10	—	50	
149	—	0·20	67	1·15
	0·20	—	77	(Average 1·14)

Excellent agreement in the potency ratio between hayatin methiodide and *d*-tubocurarine chloride was observed in different animals. The Table shows that by this method hayatin methiodide was approximately 1.14 times as potent as *d*-tubocurarine chloride in dogs.

When hayatin methiodide depressed or even abolished the contraction of the gastrocnemius muscle indirectly stimulated through the nerve, the response to direct electrical stimulation of the same muscle still persisted (Fig. 1a). This was also so with *d*-tubocurarine chloride (Fig. 1b). The amplitude of the muscle contraction to direct stimulation was, however, depressed by about 36% of the control value during complete neuromuscular blockade produced by either drug. This observation tends to show that both hayatin methiodide and *d*-tubocurarine act on the muscle directly as well as on the neuromuscular junction.

The effect of frequency of stimulation on the degree of paralysis in neuromuscular preparations has been stressed. Thus Briscoe (1936) found, with the cat femoral-quadriceps preparation, that though after tubocurarine the contraction increased in magnitude in direct proportion to the frequency of stimulation up to 240 per second, it became merely a brief twitch at the highest frequencies. With cat's sciatic-gastrocnemius preparations hayatin methiodide was found to depress the muscular contraction more with increase of frequency (0.25, 0.5, 1, 2 pulses/sec.) (Fig. 2).

The onset, development, and degree of paralysis in different muscles, after intravenous administration of hayatin methiodide to cats and dogs anaesthetized with a barbiturate, followed the same pattern as is seen with similar doses of *d*-tubocurarine chloride. As with mice and rabbits, the limb muscles were affected first and the respiratory muscles, especially the diaphragm,

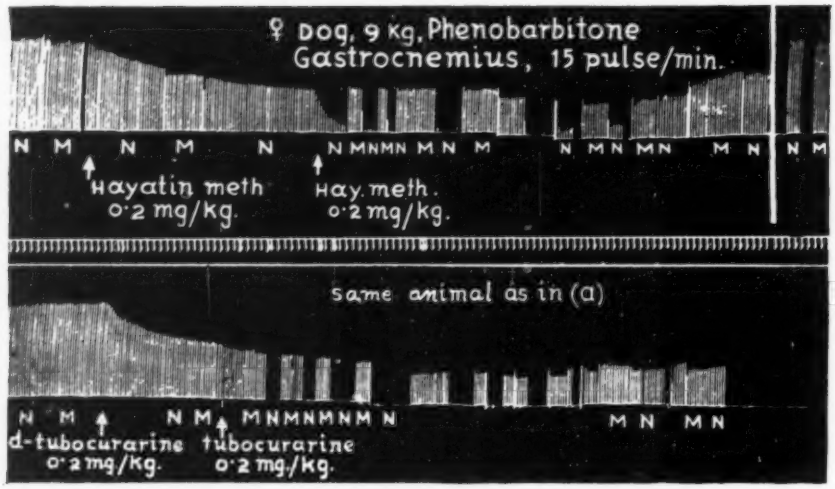

♀ Dog, 9 kg, Phenobarbitone
Gastrocnemius, 15 pulse/min.
(a)
N M N M N N M N M N M N M N M N M N M N M N
Hayatin meth 0·2 mg/kg.
Hay. meth. 0·2 mg/kg.
same animal as in (a)
(b)
N M N M M N M N M N M N M N
d-tubocurarine 0·2 mg/kg.
tubocurarine 0·2 mg./kg.

FIG. 1.—Showing depression of gastrocnemius contraction by (a) hayatin methiodide and (b) *d*-tubocurarine chloride, when the muscle is stimulated directly (M) and via the sciatic nerve (N).

last of all. The individual limb muscles also showed different degrees of susceptibility to both drugs. Simultaneous records of gastrocnemius and tibialis contractions, along with the movements of the intercostal muscles and the diaphragm, showed that *d*-tubocurarine chloride, in a dose of 0.2 mg./kg., caused 67% paralysis in the gastrocnemius, 45% in tibialis, with some stimulation of diaphragmatic movement; hayatin methiodide, in the same dose, caused 77% paralysis in both gastrocnemius and tibialis together with very slight depression of intercostal but stimulation of diaphragmatic movement (Fig. 3). Diaphragmatic movements were stimulated to a greater extent at doses which completely paralysed the skeletal muscles—probably as a compensatory measure for the depression of the intercostals. Complete diaphragmatic paralysis could, of course, be obtained with sufficiently high doses.

To find out the margin of safety for this drug, the doses causing complete paralysis of limb muscles and doses causing complete respiratory paralysis were determined. A few cats and dogs were given 0.1 mg./kg. doses every minute, until paralysis of limb muscles and of respiration was obtained. Table V gives the results of such experiments. The ratio between the two doses,

TABLE V

RATIO OF GASTROCNEMIUS AND RESPIRATION PARALYSING DOSES OF HAYATIN METHIODIDE IN CATS AND DOGS

Experiment No. and Animal Used	(1) Dose to Cause Complete Paralysis of Gastrocnemius (mg./kg.)	(2) Dose to Cause Complete Paralysis of Respiration (mg./kg.)	Ratio (2)/(1)
158-dog	0·5	0·8	1·6
159-cat	0·1	0·2	2
162-cat	0·4	0·9	2·25

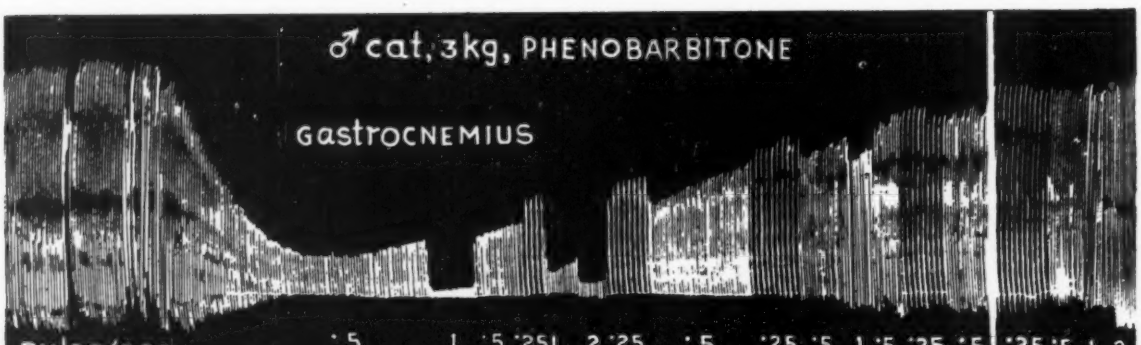
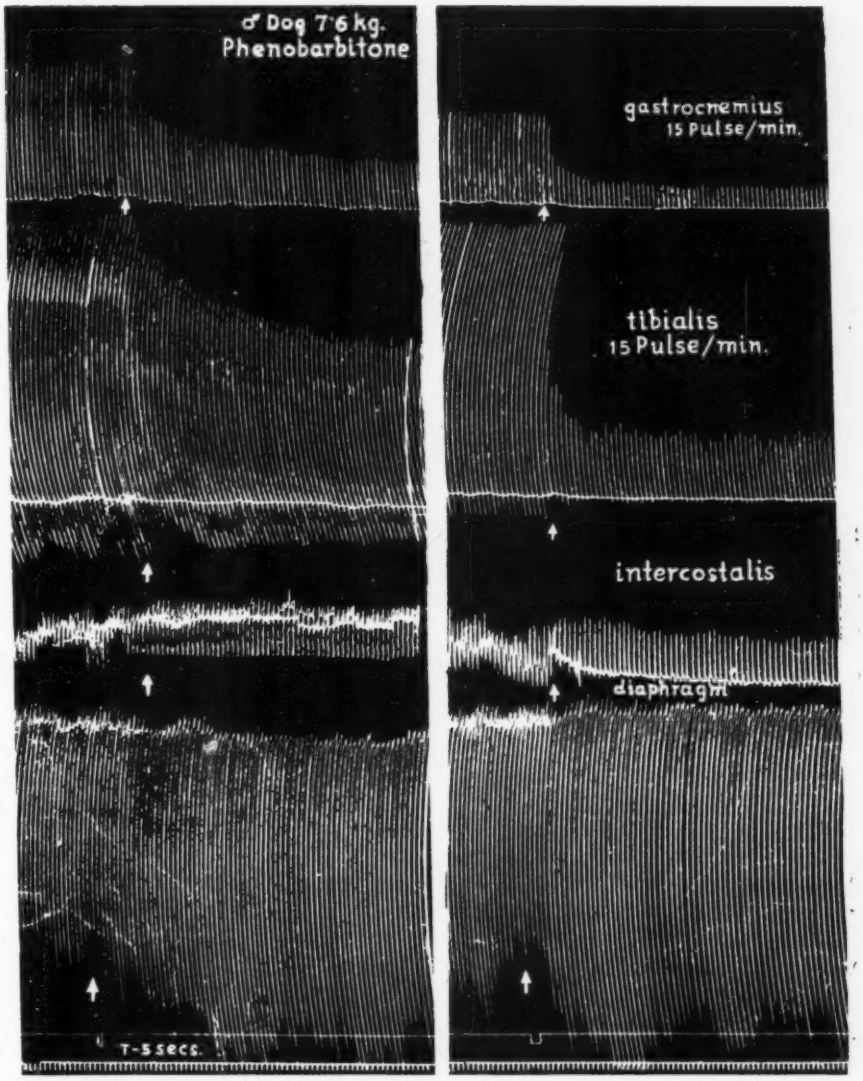


FIG. 2.—Effect of frequency of electrical stimulation on the degree of paralysis of the gastrocnemius muscle produced by hayatin methiodide. The pulse frequencies per second (0.25, 0.5, 1.0, and 2.0) are indicated on the record.

indicating the margin of safety, was about 2. This compares with a corresponding ratio of 1.25 for *d*-tubocurarine chloride and 2.2 for decamethonium iodide (Robson and Keele, 1950).

A cumulative effect of hayatin methiodide was manifested in nerve-muscle preparations of cats and dogs. Some doses of the drug were repeated in the same animal after the amplitude of muscle contraction had returned to its original level. It was found that the second dose produced paralysis of a greater degree and of a longer duration than did the first dose. The initial injection of 0.1 mg./kg. of the drug caused 71% paralysis in the gastrocnemius of a cat, with recovery after nine minutes; repetition of the same dose, however, caused 100% paralysis, with complete recovery after 13 minutes (Fig. 4).



(a) Tubocurarine chloride 0·2 mg./kg. (b) Hayatin methiodide 0·2 mg./kg.

FIG. 3.—To show variation in the response of different muscles to the action of (a) *d*-tubocurarine chloride, and (b) hayatin methiodide. Dose of each drug, 0.2 mg./kg. The two upper rows of tracing show induced contractions in gastrocnemius and tibialis muscles respectively: the two lower the spontaneous movements of intercostal and diaphragmatic muscles.

Effect of Neostigmine.—As with *d*-tubocurarine chloride, neostigmine methyl sulphate antagonized the curariform effect of hayatin methiodide. In rabbits, intravenous injection of 0.05 mg./kg. of neostigmine, one minute before the injection of hayatin methiodide, increased the HD50 from 0.0523 mg./kg. to 0.1182 mg./kg. (i.e. 2.25 times), and the LD50 from 0.0703 mg./kg. to 0.1972 mg./kg. (i.e. 2.8 times). With *d*-tubocurarine chloride the corresponding increases in the HD50 and LD50 were 1.7 and 2.1 respectively (Hoppe, 1950).

In dog-nerve muscle preparations also, neostigmine exerted its antagonistic effect on the action of hayatin methiodide. As with *d*-tubocurarine chloride, neostigmine, given in doses of 0.05–0.1 mg./kg. during curarization with hayatin methiodide, caused mainly a shortened duration of, and a more rapid recovery from, the muscular paralysis (Fig. 5). On simultaneous administration of hayatin methiodide and neostigmine, though paralysis was not completely prevented, its degree and duration were markedly diminished. Thus a dose of 0.2 mg./kg. hayatin methiodide produced 67% paralysis of the gastrocnemius muscle of a dog lasting for more than 20 minutes, whereas a combination of 0.2 mg./kg. of the drug with 0.005 mg./kg. of neostigmine produced in the same animal only 28.5% paralysis, complete recovery occurring within six minutes. Another combination, of 0.3 mg./kg. of the drug with 0.08 mg./kg. of neostigmine, caused only 30% paralysis lasting

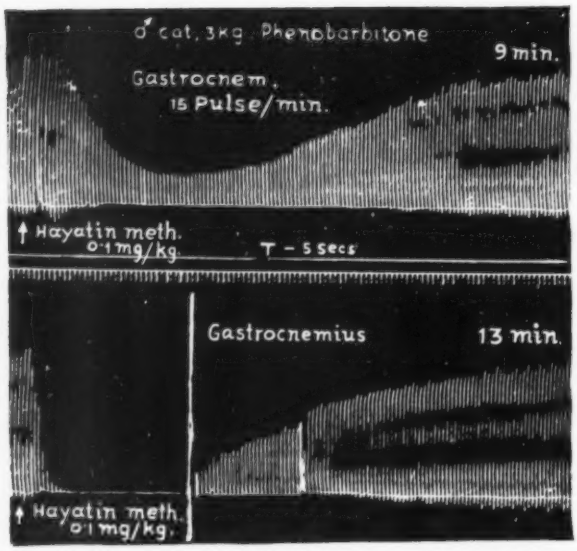


FIG. 4.—To show cumulative action of successive equal doses (0.1 mg./kg.) of hayatin methiodide on the degree and duration of paralysis of the gastrocnemius muscle. Upper record (a) shows effect of first dose; lower record (b) that of second dose. Partial paralysis in (a), with complete recovery in 9 min. Complete paralysis in (b), with recovery in 13 min.

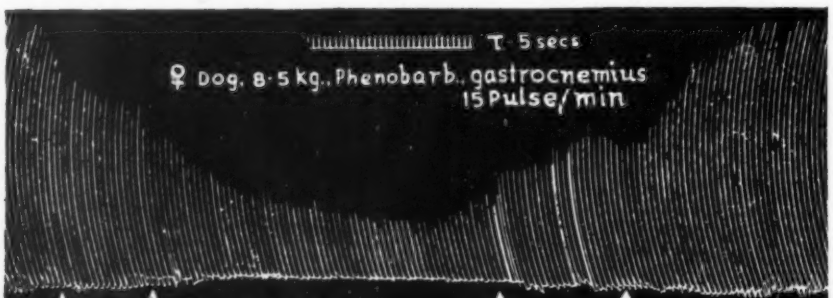


FIG. 5.—To show the antagonism of neostigmine to the curariform action of hayatin methiodide on the gastrocnemius.

for nearly 18 minutes. In another animal hayatin methiodide in a dose of 0.1 mg./kg. caused 60% paralysis of the gastrocnemius muscle, lasting for one hour. When, however, neostigmine, 0.1 mg./kg., was administered two minutes previously the paralysing effect of hayatin methiodide was completely prevented. When the hayatin methiodide was given ten minutes after the neostigmine, only 40% paralysis was obtained and recovery was quick. Neostigmine exerted a marked beneficial effect on respiration when this was seriously affected by hayatin methiodide (Fig. 6).

Previous tetanization antagonized the paralytic effect of hayatin methiodide as it does that of *d*-tubocurarine chloride.

Frog Rectus Abdominis Muscle and Antagonism to Acetylcholine.—Hayatin methiodide did not show any effect on the frog rectus abdominis even at a concentration of 1 in 10,000 (1 mg. in 10 ml.). On the other hand 0.2 mg. of hayatin methiodide could prevent the contraction produced by 0.01 mg. of acetylcholine and not more, whereas 0.1 mg. of hayatin methiodide failed to antagonize 0.01 mg. acetylcholine. Thus hayatin methiodide appeared to antagonize the action of acetylcholine on the frog's rectus though it did not itself produce any muscular contraction. In this way its action resembles that of *d*-tubocurarine chloride and not that of decamethonium.

Toxicity

Acute Toxicity.—The symptoms were more or less the same in all the species studied. Ataxia of hind limbs, incoordination of skeletal muscles, and respiratory embarrassment were the usual manifestations. When respiration became more affected, twitching or even convulsions occurred and urination followed; the animal finally died of respiratory paralysis usually within 30 to 50 minutes, rarely after an hour. At sublethal dosages, convulsive movements disappeared when respiratory movements were re-established. Salivation was noticed in some of the dogs and rabbits.

Lethal doses of hayatin methiodide in various species of animals are given in Table VI and compared with those of *d*-tubocurarine chloride.

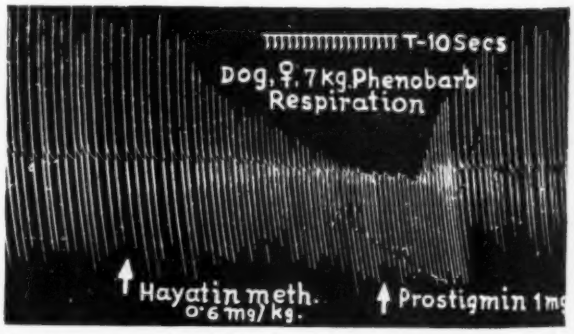


FIG. 6.—To show beneficial effect of neostigmine on respiratory movements depressed by hayatin methiodide.

TABLE VI

COMPARATIVE PHARMACOLOGICAL DATA FOR HAYATIN METHIODIDE AND *d*-TUBOCURARINE CHLORIDE

Type of Test	Hayatin Methiodide		*d*-Tubocurarine Chloride		Relative Activity of Hayatin Methiodide (*d*-Tubocurarine Chloride = 1)
	No. of Animals	Dose (mg./kg.)	No. of Animals	Dose (mg./kg.)	
Subcutaneous ED50 mice	30	0·195 ±0·0187	50	0·4 ±0·02*	2·05
,, LD50 ,,	30	0·355 ±0·0218	60	0·6 ±0·02*	1·7
Intravenous LD50 rabbits	50	0·0696±0·0022	30	0·150 ±0·0122	2·15
,, HD50 ,,	70	0·0526±0·0009	30	0·1124±0·0113	2·13
Intravenous mean head-drop dose in rabbits (by cross-over test)	10	0·0840±0·0176	10	0·1713±0·0364	2·04
Depression of nerve impulse transmission in dogs (Table IV)		— —		— —	1·14

* Figures given by Hoppe (1950).

Chronic Toxicity.—In rabbits injections of various doses—0.050, 0.052, and 0.055 mg./kg. given for five consecutive days—did not produce any untoward effect or cause significant loss of body weight.

DISCUSSION

It was reported previously (Pradhan *et al.*, 1952) that hayatin hydrochloride did not show any curariform activity even at 5 mg./kg. doses, whereas its methiodide derivative was active even at a dose of 0.1 mg./kg. The structural formula of hayatin is not known, but the methiodide probably contains a quaternary nitrogen atom and this may be responsible for the curariform activity of the compound. Though this substance works mainly by blocking transmission of nerve impulses at the neuromuscular junction, it has also some direct depressant action on the muscle. A similar property of *d*-tubocurarine chloride has been reported by certain workers (Hoppe, 1950; Acheson, 1948).

The curariform activity of hayatin methiodide seems to resemble that of *d*-tubocurarine chloride more than that of decamethonium. The characteristics are: (i) mice and rabbits show more sensitivity than cats and dogs, (ii) tibialis muscle is not more sensitive than gastrocnemius, being either equally or less sensitive, (iii) *d*-tubocurarine chloride does not antagonize it, but rather potentiates its action, (iv) neostigmine shows definite antagonism, (v) previous tetanization of the sciatic nerve antagonizes the paralysis of the gastrocnemius, (vi) it does not produce contracture on frog's rectus abdominis, but shows antagonism to acetylcholine on this muscle. Hence hayatin may be classified as a pachycurare along with *d*-tubocurarine chloride (Paton, 1951; Bovet, 1951); this might have been assumed from its occurrence in a plant of this genus of Menispermaceae.

SUMMARY

1. Hayatin methiodide causes paralysis of skeletal muscles in cats and dogs in the same manner as *d*-tubocurarine chloride.

2. In cats and dogs the curariform activity of hayatin methiodide is 1.14 times greater than that of *d*-tubocurarine chloride.

3. On the frog's rectus muscle it antagonizes acetylcholine.

4. In mice hayatin methiodide is 2.05 times as active and 1.7 times as toxic as *d*-tubocurarine chloride when given by subcutaneous injection.

5. In rabbits hayatin methiodide is 2.13 times as active as *d*-tubocurarine chloride by a quantal assay method and 2.04 times as active as the latter by a cross-over test. It is 2.15 times as toxic as *d*-tubocurarine chloride.

6. The margin of safety for both the drugs is very low.

7. The duration of curariform activity for both the compounds seems to be approximately the same.

8. Neostigmine antagonizes the curariform action of both hayatin methiodide and *d*-tubocurarine chloride.

The authors are indebted to Dr. B. Mukerji, Director, Central Drug Research Institute, Lucknow, for his interest in this work, and to Messrs. K. S. Varadan and C. Ray for their technical assistance.

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Brit. J. Pharmacol. (1953), **8**, 406.

RELEASE OF HISTAMINE BY TRYPTAMINE AND 5-HYDROXYTRYPTAMINE

BY

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(RECEIVED JUNE 17, 1953)

MacIntosh and Paton (1949) have shown that many amines have in common the property of causing histamine release. In the present experiments it will be shown that this applies to tryptamine and 5-hydroxytryptamine as well.

The following procedures were adopted for examining the histamine releasing properties of the tryptamine compounds. (1) Perfusion of isolated skin flaps and gastrocnemius muscles and assaying the histamine in the venous effluent after arterial injection of the tryptamine compounds. (2) Determining changes in histamine content of tissues after subcutaneous and intraperitoneal injections of tryptamine.

METHODS

The perfusion experiments were performed on skin flaps of the cat's and dog's hind leg and on the gastrocnemius muscle of the cat, according to the methods described by Feldberg and Paton (1951) and Feldberg and Schachter (1952). The presence in the venous samples of tryptamine and 5-hydroxytryptamine collected after their injection had to be taken into account when assaying the samples for histamine on the atropinized guinea-pig's ileum preparation, because these substances have themselves a contractile effect on this preparation. For this reason, the samples were first assayed for tryptamine or 5-hydroxytryptamine respectively on the atropinized rat's colon, according to the method described by Feldberg and Toh (1953). They were then assayed for histamine on the guinea-pig's ileum preparation by adding to the control solutions of histamine, tryptamine, or 5-hydroxytryptamine respectively. For instance, if a sample of perfusate was found to contain 100 μg. tryptamine per ml., and 0.05 ml. was required for producing a reasonable contraction of the guinea-pig's gut, the effect was compared with contractions produced by different amounts of histamine to each of which was added 5 μg. tryptamine.

Changes in histamine content of tissues after subcutaneous and intraperitoneal injections of tryptamine were studied in albino rats of about 100 g. body weight. The rats were killed by a blow on the head and samples of the tissue to be examined were removed, weighed, ground in $\frac{1}{4}$N HCl with saline solution, boiled and neutralized before testing. The method has been described elsewhere (Feldberg and Talesnik, 1953; Smith, 1953). Tissue samples from 4 to 6 rats were pooled and extracted together in order to minimize individual variations in the histamine content.

All values for histamine are expressed as base. We are indebted to Dr. R. K. Richards, Abbott Laboratories, for kindly providing us with the sample of 5-hydroxytryptamine creatine sulphate. Its molecular weight is 355, of which about half, i.e., 176, represents the base; all values given refer to the base. The tryptamine used was the hydrochloride. Its molecular weight is 196.5, of which about 80%, i.e. 160, represents the base; all values given refer to the salt. In some experiments the histamine releasing activity of tryptamine was compared with that of compound 48/80, kindly provided by the late Dr. C. H. Kellaway (Wellcome Research Laboratories).

RESULTS

Perfused Skin Flap of the Cat

Both tryptamine and 5-hydroxytryptamine caused a release of histamine from perfused skin flaps. There was, in addition, vasoconstriction and the development of oedema; the vasoconstriction, which was particularly strong after 5-hydroxytryptamine, was probably only to some degree due to the released histamine and was mainly a direct effect of the tryptamine compounds on the vessels. With the injection of either 2.5 mg. tryptamine or 5-hydroxytryptamine in 0.5 ml., the flow was either stopped or nearly stopped by a vasoconstriction which supervened about 2 min. after the injection. When the flow was not totally arrested, the vasoconstriction was found to reach a maximum in 5–7 min. and then very gradually subsided. The intensity of the oedema paralleled the output of histamine; it developed during the first 30 or 60 min. after the injection, was particularly pronounced in the subcutaneous tissue, and

*During tenure of a Fellowship from the Medical Research Council.

could amount to about six times the weight of the skin flap.

As was to be expected, the first samples after the injections contained most of the tryptamine or 5-hydroxytryptamine. They were recovered in the venous effluent to between 50–70%. Since the first drops of venous effluent after the injection, which probably contained the highest concentration of the tryptamine compounds, were discarded, the actual amounts escaping into the venous effluent were probably greater. Further, some destruction and inactivation of these compounds may have occurred during the passage through the skin and some may have been retained, as evidenced by their presence in the oedema fluid collected after cessation of the perfusion.

Table I shows the amounts of tryptamine and 5-hydroxytryptamine from skin flaps of the same cat in successive samples of venous effluent, and the histamine content of these samples when assayed against known histamine solutions but incorporating the amounts of the tryptamine compounds present in them.

TABLE I

ASSAY OF VENOUS EFFLUENT FOR TRYPTAMINE (T), 5-HYDROXYTRYPTAMINE (HT) AND HISTAMINE (Hi) FROM PERFUSED CAT'S SKIN FLAPS OF THE SAME CAT

2 mg. T injected into the right, and 2 mg. HT into the left skin flap (same experiment as No. 9, Table II)

Sample after Injection	Minutes of Collection	Right Skin Flap			Left Skin Flap		
		ml. Collected	μg. T	μg. Hi	ml. Collected	μg. HT	μg. Hi
1	1½–2	3·0	525	36·5	2·2	660	79·2
2	8–8½	4·4	396	145·2	1·3	78	35·8
3	10	5·0	100	25·0	11·4	160	43·3
4	20	22·0	143	66·0	15·0	63	60·0
5	10	14·7	28	7·4	12·7	13	0·6
6	10	13·5	11	1·4	20·5	17	0·8
7	20	24·2	—	1·6	16·5	12	0·5
8	20	15·0	—	0·5	—	—	—
Oedema fluid		75·0	75	7·5	68·0	54	7·2
Total ..			1,273	291·1		1,057	227·4

Table II shows the amounts of histamine released in eleven experiments on injection of 0.5–2 mg. of the two tryptamine compounds. Doses under 0.5 mg. were ineffective, but with 2 mg. usually between 93 and 283 μg. histamine was released. The low value of 30 μg. in experiment 6 represents only the histamine of the sample collected during the first 5 min. after the injection; in this experiment the vasoconstriction was so intense that the flow stopped after this time and attempts to restart it by increasing the perfusion pressure led to leakage from the arterial side, so that the experiment had to be discontinued.

In experiments 4, 5, 8, and 9 of Table II, the skin flaps of both hind legs were perfused and

TABLE II

OUTPUT OF HISTAMINE AFTER TRYPTAMINE (T) AND 5-HYDROXYTRYPTAMINE (HT) FROM PERFUSED SKIN PREPARATIONS OF THE CAT

Figures in brackets refer to the highest histamine concentration (μg./ml.) found in the initial samples after injection of the tryptamine compounds

Expt. No.	mg. Injected	Output of Histamine in μg.			
		After (T)		After (HT)	
1	0·5	1	(0·09)	—	—
2	0·5	—	—	28·5	(2)
3	1·0	11·2	(1·05)	—	—
4	1·0	46·8	(14)	68	(20)
5	2·0	93	(4)	115	(8)
6	2·0	106	(5)	30*	(6)
7	2·0	172	(20)	—	—
8	2·0	187	(32)	127	(28)
9	2·0	283	(33)	220	(36)
10	2·0	—	—	172	(20)
11	2·0	229	(22)	—	—

* Only one 5 min. sample collected.

tryptamine was examined on one side and the equivalent amount of 5-hydroxytryptamine on the other. It is evident that the two compounds have approximately the same histamine releasing ability; if there is any difference, tryptamine is slightly more active. Since we express 5-hydroxytryptamine as base and tryptamine as salt, 2 mg. of tryptamine corresponds to 1.6 mg. of base only, and the difference between the activity of the two compounds is thereby accentuated.

A similar conclusion is reached from experiments in which the effects of repeated injections are compared on the same skin preparation. In the experiments of Fig. 1, 2 mg. of 5-hydroxytryptamine and 2 mg. of tryptamine were injected into the skin flaps of both legs of the same cat, but in reversed order. In both perfusions the first injection, whatever the tryptamine compound injected, produced a greater histamine output than the subsequent injection. It was possible, however, to increase the histamine output considerably by doubling the dose injected. This is seen for tryptamine at the end of the perfusion of the left skin flap (Fig. 1).

The vasoconstriction interfered with the time course of the appearance of histamine in the venous outflow, and the trough seen in the record of the histamine output after 4 mg. tryptamine is explained by the fact that during the corresponding period the flow was greatly reduced and therefore only a small volume of venous effluent collected. The vasoconstriction interfered also with the output of histamine during some of the previous injections in the experiment of Fig. 1, by delaying the maximal output per minute until the vasoconstriction had subsided. However, the actual " release " of histamine probably occurs as

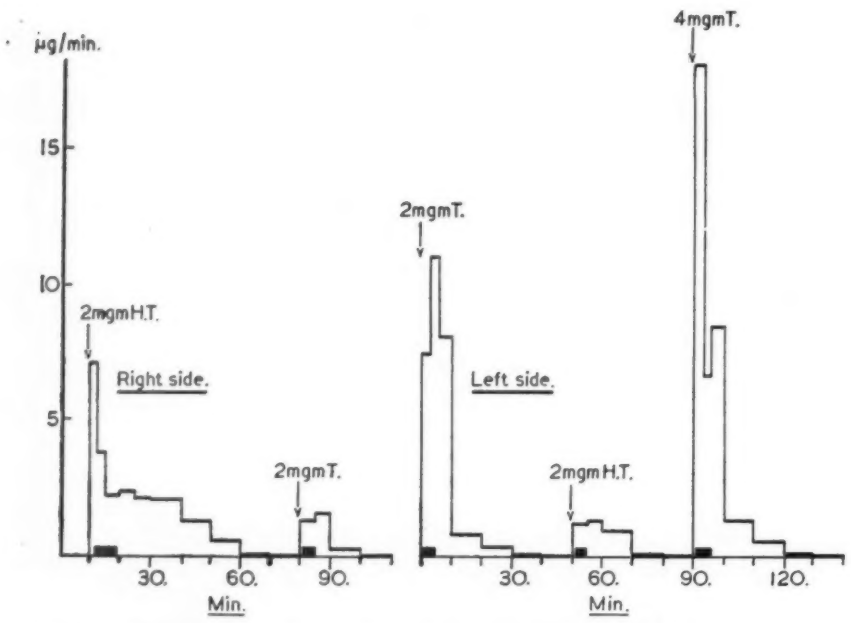

FIG. 1.—Histamine output from perfused skin preparations of the hind legs of the same cat after tryptamine (T) and 5-hydroxytryptamine (HT). The black areas above the base-line indicate periods of pronounced vasoconstriction. (For details see text.)

skin preparations from the same cat in which the order of injection was reversed. The tryptamine compounds are therefore about as active as, or perhaps even slightly more active than, propamidine. They are certainly more active than the other amines and amidines examined by MacIntosh and Paton (1949), and also more active than the opium alkaloids and morphine derivatives examined by Feldberg and Paton (1951).

Perfused Gastrocnemius Muscle of the Cat

The histamine releasing activity of tryptamine and 5-hydroxytryptamine from the perfused gastrocnemius muscle is illustrated in the two experiments of Fig. 3, which are from different cats. Since the gastrocnemius has a low histamine content, the amounts released from this prepara-

instantaneously as after the other known histamine liberators.

The histamine release was associated with a reduction in the histamine content of the central parts of the perfused skin. In one experiment a release of 173 μg. histamine after 2 mg. tryptamine reduced the histamine content in the central part of the skin flap by 75%, to 2.9 μg./g.; in a corresponding experiment with 2 mg. 5-hydroxytryptamine a release of 171 μg. histamine caused a reduction by 88%, to 2.6 μg./g.

When the histamine releasing property of the two tryptamine compounds is compared on the perfused cat's skin preparation with that of 48/80, it is found that, weight for weight, 48/80 is about 100 times more active. This is illustrated in the experiment of Fig. 2, in which the effect of 20 μg. 48/80 is compared with that of 2 mg. tryptamine, on two

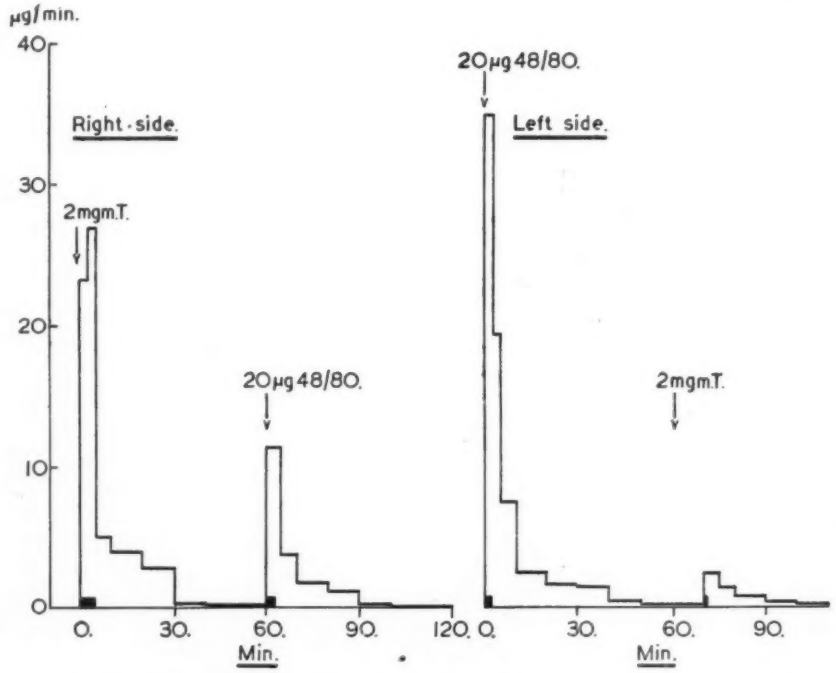

FIG. 2.—Comparison of the histamine output by 48/80 and tryptamine (T) from perfused skin preparations of the hind legs of the same cat. The black areas above the base-line indicate periods of pronounced vasoconstriction. (For details see text.)

tion are much smaller than those released from the cat's skin. In the first experiment, two successive injections of 2 mg. 5-hydroxytryptamine released 3.35 and 0.71 μg. histamine ; in the second experiment 2 mg. tryptamine released 2.59 μg. histamine, and a succeeding injection of 4 mg., 6.35 μg. Although the two experiments were from different cats, they suggest that for the perfused gastrocnemius muscle, as for the perfused skin of the cat, the histamine releasing property of the two compounds is of the same order.

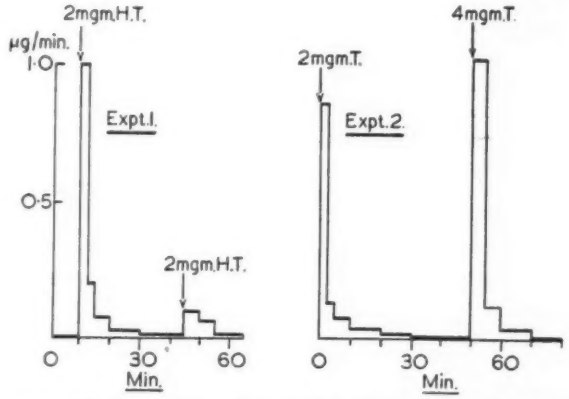

FIG. 3.—Histamine output from perfused gastrocnemius after 2 mg. 5-hydroxytryptamine (HT) in expt. 1 and after 2 and 4 mg. tryptamine (T) in expt. 2 which was from a different cat.

The amounts of histamine released in the two experiments of Fig. 3 represent only a fraction of the muscle histamine. In the experiment with 5-hydroxytryptamine, the gastrocnemius of the non-perfused leg weighed 30 g. and contained 1.38 μg./g. histamine ; its total histamine content was therefore about 41 μg. ; the amounts released by the two injections of 5-hydroxytryptamine correspond, therefore, to about 10% of this figure. In the other experiment, the weight of the non-perfused gastrocnemius was 28.5 g. and contained 1.64 μg./g. histamine ; its total histamine content was therefore about 47 μg., of which a little over 20% was released by the two tryptamine injections.

Perfused Skin Flap of the Dog

Tryptamine alone was examined, which produced some vasoconstriction, but to a less degree than on the cat's skin preparation. When assaying the effluent for tryptamine on the rat's colon, the values obtained for the combined first three samples, collected over a 10 min. period after the injection, were higher than the actual amounts of tryptamine injected. This may have resulted from the fact that these samples collected during the vasoconstriction were blood-stained, even if the effluent had become clear before the injection ; and it had been found that samples collected at the beginning of a perfusion, when they were yet blood-stained and cloudy but no tryptamine had been injected, had some activity on the rat's colon. It was not examined whether this activity was due to tryptamine or 5-hydroxytryptamine. In order to avoid the error of exaggerating, by this activity, the histamine assayed in the effluent, two methods were adopted : one (method 1) by taking the excessively high value actually assayed for tryptamine on the rat's colon as representing the tryptamine in the sample, and the other (method 2) by assuming that the first three samples contained the total amount of tryptamine injected. With both methods a histamine release could be demonstrated, but, as seen from Table III, the values

TABLE III

HISTAMINE OUTPUT BY TRYPTAMINE FROM PERFUSED SKIN PREPARATION OF THE DOG

Tryptamine Injected	μg. Histamine Assayed	
	By Method 1	By Method 2
2 mg. in 0·5 ml.	7·0	—
4 ,, ,, 2 ,, ...	5·3	5·8
8 ,, ,, 2 ,, ...	6·8	9·6

obtained with the second method were higher ; even these may be an underestimation of the actual amounts of histamine present in the samples, because it is unlikely that there was a 100% recovery of tryptamine.

Histamine Release from Rat's Tissue by Tryptamine

Acid saline extracts from rat tissue produced contraction of the rat's colon ; the activity corresponded to that of 10–20 μg. tryptamine per gramme tissue. Somewhat higher values were obtained from the animals treated with tryptamine. Although such amounts of tryptamine would not greatly interfere in the histamine assay, they were taken into account by first assaying the extracts against tryptamine on the rat colon, and, in the subsequent histamine assay on the guinea-pig's ileum, adding tryptamine to the control solution of histamine, as in the corresponding assays of perfusate (see Methods).

Subcutaneous Injections.—As with 48/80, the subcutaneous injection of tryptamine leads to a reduction of skin histamine at the site of injection. For this purpose 2 mg. tryptamine was injected in 0.5 ml. in previously demarcated areas of the abdominal skin in eight rats. The injections

caused locally a bluish cyanosed area surrounded by erythema which two hours later became cyanosed also. By this time a haemorrhagic weal had developed at the injection site. Half of the injected rats were killed two hours after the injection; the skin at the site of the injection contained blood cells and plasma. The other half of the rats were killed 24 hours after the injection; the site of injection was no longer oedematous but contained a crust of cells and dried serum. The histamine content of the skin areas at the site of injection from these rats is given in Table IV

TABLE IV

EFFECT OF SUBCUTANEOUS INJECTION OF 2 MG. TRYPTAMINE IN 0·5 ML. ON HISTAMINE CONTENT OF ABDOMINAL SKIN (POOLED SKIN SAMPLES) AT SITE OF INJECTION

No. of Rats	Treatment	μg./g. Histamine	% Reduction
2	None	50	0
2	2 hr. after injection of saline ..	42	16
4	2 ,, ,, ,, ,, tryptamine	14	72
4	24 ,, ,, ,, ,, ,,	12·8	74

and compared with the histamine content from normal rats and from skin areas of rats having received a control injection of 0.5 ml. saline solution. Tryptamine produces a great reduction in histamine content which persists after all traces of oedema have disappeared. The small reduction two hours after the saline injection must be accounted for by an increased fluid content of the skin due to the fact that the injected fluid was not completely absorbed.

There is this difference between the effects of tryptamine and those of corresponding experiments with 48/80 (see Feldberg and Talesnik, 1953) that the injection of tryptamine, probably through its intense vasoconstrictor properties, produces local damage to the tissue which may contribute to the observed reduction in histamine content.

Intraperitoneal Injections.—Eight rats were each given a total of 13 mg. of tryptamine intraperitoneally. The injections were made twice daily, starting with 1 mg. and going up to 4 mg., as shown in Table V.

After the injection of 1 mg., respiration became accelerated, and after 2 and 3 mg. was, in addition, laboured. The rats became cyanotic, their coat was ruffled, but later the skin became pink, and in some animals oedema developed round the mouth and on the paws. This occurred after 2 mg. in three, and after 3 mg. in five out of eight rats about 45 min. after the injections. With 4 mg. the animals were first prostrated, cyanosed, and cold, and oedema developed in half the animals when they began to recover.

Table V gives the histamine content of various tissues from these animals killed 24 hours after the last injection. The values from the pooled samples are compared with those obtained from six control rats. There is a definite reduction in skin histamine and also, to a lesser degree, in the histamine content of the diaphragm, but the histamine content of the viscera showed no consistent

TABLE V

CHANGES IN HISTAMINE CONTENT OF RAT'S TISSUES AFTER INTRAPERITONEAL INJECTIONS OF TRYPTAMINE (FIRST DAY 1 MG. TWICE; SECOND DAY, 2 MG. TWICE; THIRD DAY, 3 MG. ONCE AND 4 MG. ONCE)

Area	μg./g. Histamine		% Change after Tryptamine
	Controls	After Tryptamine	
Skin of ear	50	35	−30
Skin of paw	55	30	−46
Skin of abdomen ..	32	18	−39
Diaphragm	11·5	8·5	−26
Liver	0·7	0·7	0
Lung	1·0	0·9	−10
Aorta	2·3	2·5	+9
Stomach	12·5	11·5	−8

change. The difference of 10% or less cannot be regarded as sufficient proof for an effect of the tryptamine.

DISCUSSION

The finding that tryptamine and 5-hydroxytryptamine have the ability to release histamine from living tissue brings them into line with the large group of amines having this property. Although the activity of tryptamine and 5-hydroxytryptamine in releasing histamine is somewhat greater than that of the other amines and amidines examined by MacIntosh and Paton (1949), and is, in fact, slightly more active than that of propamidine, the most active compound examined by these authors, this action must, nevertheless, be regarded as a side-effect of their pharmacological properties. This does not eliminate the possibility that histamine release is at the root of certain phenomena, seen after injection of tryptamine compounds and resembling histamine effects; for instance, it may account for some of the depressor effects on the arterial blood pressure obtained with 5-hydroxytryptamine in cats and dogs (Erspamer, 1952; Page, 1952; Freyburger, Graham, Rapport, Seay, Govier, Swoap, and Vander Brook, 1952) and is most likely the cause of the intense vasodilatation associated with itching seen in the rat after subcutaneous injection of a large dose of 5-hydroxytryptamine (Erspamer, 1952).

On the other hand, histamine release may occur without its usual signs, because the strong vasoconstrictor action of tryptamine or 5-hydroxytryptamine may override a histamine vasodilatation.

Recent work has shown that 5-hydroxytryptamine is a natural constituent of some animal tissues (Rapport, 1949 ; Erspamer and Asero, 1952 ; Bacq, Fischer, and Ghiretti, 1952 ; Dalgliesh, Toh, and Work, 1953 ; Feldberg and Toh, 1953) ; if released in the body it might well, in its turn, cause release of histamine. Although there is as yet no evidence that this sequence of events occurs in the body, the possibility cannot be excluded that such a mechanism participates in the histamine release by peptone and by the antigen-antibody reaction of anaphylaxis, because 5-hydroxytryptamine is released from platelets by the antigen-antibody reaction *in vitro* (Humphrey and Jaques, 1953) and because release of histamine from the dog's liver and the rabbit's lung by either antigen or peptone is, to a great extent, dependent on disintegration of platelets (Dragstedt, 1941 ; Rocha e Silva, 1950). Even if, under these special conditions, the release of 5-hydroxytryptamine from platelets by antigen or peptone should be an intermediate step for the release of histamine, not only from the platelets themselves, but perhaps also from the liver and lung cells with which the agglutinated platelets come in close contact when they are " trapped " in the capillary bed of these organs, 5-hydroxytryptamine is probably not the only intermediate histamine liberator set free. It is also necessary to keep in mind, in this connection, that the symptomatology following injections of 5-hydroxytryptamine does not resemble closely that of anaphylaxis.

Histamine is also released from the platelets during clotting (Code, 1952), which is, moreover, associated with release into the serum of 5-hydroxytryptamine (Rapport, Green, and Page, 1948 ; Rapport, 1949 ; Reid and Rand, 1951). The release of histamine in this process may again be subsequent to that of 5-hydroxytryptamine and, during intravascular thrombosis, release of 5-hydroxytryptamine from the thrombus may, in turn, lead to release of histamine from the endothelial lining of the vessel.

Summary

Tryptamine and 5-hydroxytryptamine release histamine from living tissue ; this finding brings them into line with the large group of amines having this property. The histamine-releasing activity of tryptamine and 5-hydroxytryptamine is about 100 times less than that of compound 48/80. The release of histamine was demonstrated under the following experimental conditions :

(*a*) After arterial injection of either tryptamine or 5-hydroxytryptamine into the perfused skin flap or gastrocnemius muscle of the cat.

(*b*) After arterial injection of tryptamine into the perfused skin flap of the dog.

(*c*) After subcutaneous or repeated intraperitoneal injections of tryptamine to rats. The subcutaneous injection caused a local reduction in the histamine content of skin at the site of injection, the intraperitoneal injections a general reduction of the histamine content in skin and skeletal muscle.

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Brit. J. Pharmacol. (1953), **8**, 412.

ANAPHYLAXIS AND HISTAMINE RELEASE IN THE RABBIT

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(RECEIVED JUNE 24, 1953)

Since the time that the similarity of the symptoms of histamine intoxication and acute anaphylactic shock was pointed out by Dale and Laidlaw (1910), an impressive body of evidence implicating histamine in anaphylaxis has accumulated. An increased concentration of circulating histamine was obtained by Dragstedt and Gebauer-Fuelnegg (1932) in supradiaphragmatic venous blood of the shocked dog. Similar observations were later made for the guinea-pig as well as for the dog (Code, 1939). The release of histamine from an isolated tissue during the antigen-antibody reaction of anaphylaxis was shown for the perfused guinea-pig lung (Bartosch, Feldberg, and Nagel, 1932), and Schild (1939), employing a tissue incubation technique, found that this was true, though to a varying degree, for many tissues of the guinea-pig. The anaphylactic release of histamine is marked in the liver of the dog (Dragstedt and Gebauer-Fuelnegg, 1932; Dragstedt and Mead, 1936; Rocha e Silva, Scroggie, Fidler, and Jaques, 1947), and, as has recently been demonstrated, also occurs in the isolated, perfused skin of this species (Feldberg and Schachter, 1952).

The above observations, along with others, support the hypothesis that the liberation of histamine is an important accompaniment of anaphylactic shock. The released histamine, through its effects at the site of release plus its subsequent systemic actions, can to some extent account for the toxicology of the immediate anaphylactic reaction in the guinea-pig and dog. Observations in other species, however, have raised some doubt about the "fundamental" role of histamine in anaphylaxis (see Ratner, 1943). For example, although histamine is released into the plasma from formed elements of rabbit blood during the antigen-antibody reaction *in vitro* (Katz, 1940; Dragstedt, Ramirez de Arellano, Lawton, and Youmans, 1940; Rose and Browne, 1941; Carryer and Code, 1950), in the intact rabbit, anaphylaxis is regularly accompanied by a marked reduction in the concentration of histamine in whole blood (Rose and Weil, 1939; Rose, 1941). Code and Hester (1939) likewise found a reduction in blood histamine concentration during the anaphylactic reaction in the horse and calf.

The present experiments were primarily designed to investigate whether direct evidence for the local release of histamine during the antigen-antibody reaction in various rabbit tissues could be obtained. They revealed that this does indeed occur, to a marked degree from skin, also from liver, and to a lesser degree from small intestine.

The possibility that other pharmacologically active substances might be released, or formed, during the anaphylactic reaction in the rabbit was also considered, since there is evidence that smooth muscle stimulating substances other than histamine may appear in the blood. For example, bradykinin, a substance producing a slow contraction of the guinea-pig ileum (Rocha e Silva, Beraldo, and Rosenfeld, 1949), has been shown to appear in the blood of dogs during anaphylaxis (Beraldo, 1950). The possibility that 5-hydroxytryptamine (serotonin, thrombotonin) might be released from platelets during the antigen-antibody reaction has been suggested by Rand and Reid (1952). Recent *in vitro* studies on blood using anti-platelet serum have revealed the release of a smooth muscle stimulating substance other than histamine or bradykinin (Moussatche and Cruz, 1952). Also, Humphrey and Jaques (1953) have reported the appearance of a substance resembling 5-hydroxytryptamine from rabbit blood during the antigen-antibody reaction *in vitro*. The present experiments, using the guinea-pig ileum as a test preparation, failed to detect the release of a smooth muscle stimulating substance other than histamine during the antigen-antibody reaction in isolated perfused skin, liver, or intestine.

MacIntosh and Paton (1949) commented on the similarity of anaphylaxis and the result of administration of histamine releasing substances, and

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Mongar and Schild (1952) have since demonstrated a parallelism in the relative effectiveness of histamine releasing compounds such as 48/80 and curare with that of antigen in releasing histamine from guinea-pig tissues. These observations are of interest in so far as they suggest that a histamine releaser may be produced by the antigen-antibody reaction. The present experiments, however, demonstrate a discrepancy in the histamine releasing ability of these agents in the perfused rabbit liver; large amounts of 48/80 were ineffective, whereas antigen regularly released considerable amounts of histamine from sensitized preparations.

METHODS

White New Zealand rabbits (2.5–4 kg.) were sensitized by 6 daily intraperitoneal injections of 1 ml. horse serum and used for experiment in from 10 days to 6 weeks after the last injection. Anaesthesia was accomplished by intravenous sodium pentobarbitone (30 mg./kg.) plus ether if necessary. Arterial blood pressure was recorded from the carotid artery. Blood samples for plasma histamine assay were obtained in the following manner. Blood was drawn into a heparinized syringe with as little suction as possible and gently introduced into centrifuge tubes containing heparin. In later experiments syringes and centrifuge tubes were siliconed to reduce further the degree of "spontaneous" histamine release from the blood cells (see Code, 1952). The blood was immediately centrifuged at 3,000 rev./min. for 10 minutes, the plasma removed and assayed at once. In the dye experiments, a 5% solution of pontamine sky blue in saline was injected in a dose of 1 ml./kg., somewhat less than that used by Miles and Miles (1952) in studying permeability changes in guinea-pigs.

Perfusion of Isolated Skin.—The entire ear was removed after cutting away excess fur, and clipped on to a sloping square piece of glass. The perfusion system consisted of one or two elevated reservoirs containing the solutions to be perfused, which were continually aerated with 95% oxygen and 5% carbon dioxide. Polythene tubing led from the reservoir to a Murphy drip which permitted observation of the rate of inflow. Another long strip of polythene tubing connected the Murphy drip to a glass T tube. A short piece of rubber pressure tubing connected the T tube to the arterial cannula, a blunted hypodermic needle. Care was taken to remove air bubbles from the system, and the needle cannula was inserted into the central artery of the ear. After the flow was started the cut ends of the small veins of the ear were again cut with fine scissors to assist the flow and expel small clots. Single intra-arterial injections of 48/80 were made through the rubber tubing and the injected material was allowed to remain in contact with the ear tissue for 10–20 sec. by clamping off the inflow from the reservoir for this interval. The side arm of the glass T tube served as an air trap from which small bubbles could be removed if necessary. The perfusate ran along the margin of the supporting glass plate and was collected as it dropped. The rate of flow was regulated by a screw clamp on the polythene tubing so that it ranged from 18 to 25 ml./min. In those instances where the perfusion was altered from Locke solution to antigen (2% horse serum in Locke solution), two reservoirs were required and a glass Y tube was inserted in the system near the T tube (towards the reservoir). By opening the arm of the T tube the dead space fluid could be quickly removed on transferring from Locke solution to antigen. At the same time the polythene tubing connected to the Locke solution reservoir was completely clamped off. During the experiment the temperature of the skin of the ear was kept at 30–35° C. by a lamp. The fluid in the reservoir was usually kept at room temperature; in a few instances it was maintained at 35–40° C.

Perfusion of the Isolated Liver.—The arrangement of reservoirs, etc., was as for the ear perfusion. The liver, however, was perfused *in situ* and a glass instead of a needle cannula inserted into the portal vein. The abdomen was opened by a long mid-line incision, the intestine wrapped in a warm damp towel and pushed to one side. The portal vein was freed from the surrounding tissue and loose ligatures placed around it in preparation for cannulation. Small veins entering the portal vein superior to the loose ligatures were ligated. The chest was opened in the mid-line, retracted, and the thoracic inferior vena cava freed and prepared for cannulation. Artificial ventilation was begun after the chest was opened. The abdominal inferior vena cava was tied off above the right renal vein. The portal vein was then cannulated and perfusion begun. The thoracic aorta was quickly tied off and the inferior vena cava in the chest cannulated. The perfusate soon became clear and collection was begun. An abdominal pocket was built up around the liver with cotton-wool and the liver was immersed in warm paraffin which was kept at 35–40° C. by means of a lamp. The outflow was regulated to range between 25 and 35 ml./min.

Perfusion of Isolated Intestine.—A strip of upper intestine 18–30 in. in length (measured with mesentery removed after the experiment) was separated from the rest of the intestinal tract; its mesentery and blood supply were kept intact, and the superior mesenteric artery and portal vein were dissected free from surrounding tissue. The remainder of the intestinal tract was removed. The isolated loop of intestine was allowed 15–30 min. to recover from the operative procedure. Subsequently, glass cannulae were inserted into the mesenteric artery and portal vein, and the intestinal preparation was removed from the animal and transferred to a funnel-shaped bath maintained at 37° C. by a water jacket. The outflow of perfusate was regulated to 15–25 ml./min. In other respects the perfusion apparatus was the same as that used for skin and liver.

Histamine Assay.—This was performed on the isolated atropinized guinea-pig ileum preparation in an 18-ml. bath of magnesium-free Tyrode solution. 0.1 μg. atropine was added to the bath after each test. Abolition of the contraction by similar addition of 0.4 μg. of the antihistamine drug mepyramine served to identify the active substance as histamine. Those perfusates which contained 2% horse serum were assayed against histamine made up in Locke solution containing this concentration of horse serum, and equal volumes of the histamine standard and test solutions were added to the bath during assay. This precaution was taken, since histamine in 2% horse serum produced a smaller contraction than the same concentration of histamine in saline or Locke solution.

Histamine Extraction from Rabbit Ear.—The entire skin of the ear was easily stripped from the cartilage, weighed, finely divided with scissors and the histamine extracted by grinding with sand and hydrochloric acid as described by Douglas, Feldberg, Paton, and Schachter (1951). Since the cartilage contained very little histamine (<1.0 μg./g.), the histamine released into the perfusate can be considered as originating almost entirely from skin.

Drugs.—Histamine was used as the acid phosphate, atropine as sulphate, and mepyramine as maleate. Compound 48/80, a condensation product of *p*-methoxyphenethyl-methylamine with formaldehyde (Baltzly, Buck, de Beer, and Webb, 1949), was employed as a histamine liberator because of its relatively high potency in this respect (Paton, 1951). 5-Hydroxytryptamine was in the form of the creatinine sulphate and was kindly supplied by Dr. R. K. Richards of Abbott Laboratories, Chicago. Since serotonin (or thrombotonin) has been identified as 5-hydroxytryptamine (Rapport, 1949) the chemical term is employed for this substance. All values for histamine and 5-hydroxytryptamine are expressed as base.

Results

Release of Histamine from the Skin of the Ear.—Experiments were performed on ear preparations from five sensitized rabbits and five similar experiments were carried out on ears from normal rabbits. The perfusion was started with Locke solution and was transferred to antigen (2% horse serum in Locke) after one or two 10 minute samples of perfusate had been collected. During perfusion with Locke solution the concentration of histamine in the perfusate from ear preparations of normal or sensitized rabbits was never greater than 0.01 μg./ml., and became undetectable after 10 minutes. Similarly, antigen failed to release histamine from ears of normal rabbits. In preparations from sensitized rabbits, however, perfusion with antigen resulted in the release of large amounts of histamine in every instance. The total amounts of histamine released in these experiments ranged from 22.8 to 41.5 μg., the detailed results of which are shown in Table I. The time course of the release in a typical experiment is shown in Fig. 1. Fig. 2 is a record of actual contractions of the guinea-pig ileum produced by successive perfusate samples before and during perfusion with antigen. It also shows the complete abolition of the activity of the samples by mepyramine.

TABLE I

THE RELEASE OF HISTAMINE FROM ISOLATED EARS OF SENSITIZED RABBITS ON PERFUSION WITH ANTIGEN (2% HORSE SERUM)

Exp. No.	Total Histamine in Perfusate from "Shocked" Ear (μg.)	Total Histamine Residue in "Shocked" Ear (μg.)	Total Histamine Released + Residue in "Shocked" Ear (μg.)	Total Histamine Extracted from Unperfused Ear (μg.)
1	34·7	89·6	124·3	132·0
2	22·8	96·0	118·8	131·2
3	34·9	68·9	103·8	127·3
4	41·5	177·8	219·3	216·0
5	37·4	92·5	129·9	144·6

In the experiments on ears from sensitized rabbits the histamine remaining in the skin of the ear was extracted after perfusion with antigen. Since the total histamine released into the perfusate was known it was possible to compare the total extractable histamine from the unperfused ear

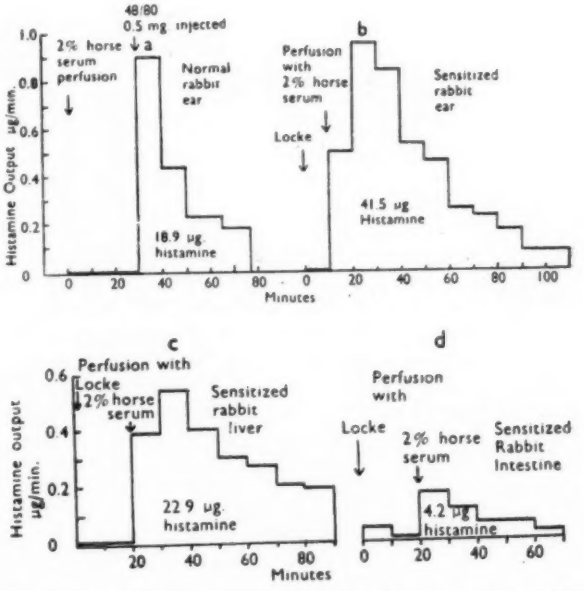

FIG. 1.—(*a*) Output of histamine from isolated ear of normal rabbit following a single intra-arterial injection of 0.5 mg. 48/80. The perfusion fluid in this experiment is 2% horse serum, which is completely ineffective in releasing histamine from the ear of a non-sensitized rabbit. (*b, c, d*) Output of histamine from isolated ear, liver, and intestine of sensitized rabbits on transferring perfusion from Locke solution to 2% horse serum (antigen).

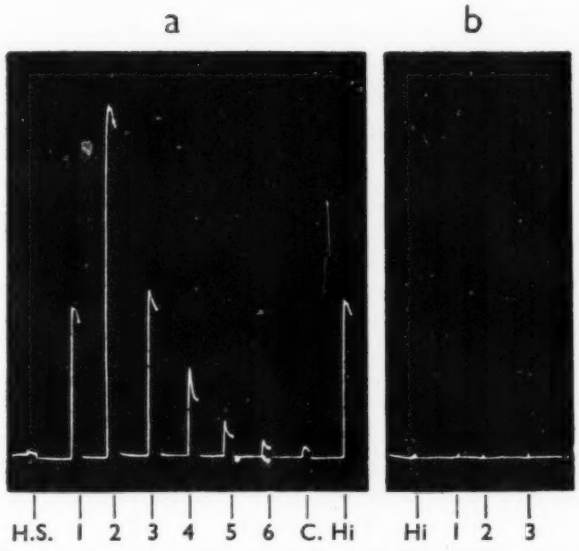

FIG. 2.—Contractions (15 seconds) of isolated guinea-pig ileum produced by perfusates from ear of sensitized rabbit obtained before and during perfusion with 2% horse serum (antigen). Between (*a*) and (*b*) 0.4 μg. mepyramine added to bath. (*a*) H.S., 0.5 ml. 2% horse serum. C., control, 1.0 ml. Locke perfusate before perfusion with horse serum. Hi, 0.1 μg. histamine in 2% horse serum. 1–6, 0.25 ml. perfusate of successive 10-minute samples during perfusion with 2% horse serum. (*b*) Hi, 0.2 μg. histamine. 1–3, 1.0 ml. of samples 1, 2, and 3 as in (*a*).

with the sum of that released from the "shocked" ear plus the amount subsequently extractable from it. The results indicate that the amount of histamine released from the skin of the rabbit's ear by antigen plus that remaining after the reaction corresponds to the total extractable histamine (Table I). The latter was, in most instances, slightly greater. This is probably due to some destruction of histamine during its release and subsequent collection. A similar conclusion regarding the pre-formed state of histamine released by antigen was deduced by Bartosch (1935) from experiments on perfused guinea-pig lung.

Single intra-arterial injections of 0.05–2.0 mg. compound 48/80 in 0.5 ml. saline regularly released histamine from Locke-perfused ears of normal rabbits. Repeated injections of the same amount of 48/80 released only small additional amounts. The amounts of histamine released in eleven experiments are shown in Table II. Except for a single experiment, where the injection of 0.5 mg. 48/80 released 82.0 μg. histamine, perfusion with antigen was more effective than 48/80 in releasing histamine. This is a reversal of the relative effectiveness of these two means of releasing histamine from dog skin (Feldberg and Schachter, 1952). As in the dog, however, the release by antigen was slower in onset and more protracted than by 48/80, and small amounts of histamine were still being released at a low but maintained concentration even after two hours of perfusion with antigen (Fig. 1).

TABLE II

HISTAMINE RELEASE ON INTRA-ARTERIAL INJECTION OF COMPOUND 48/80 IN THE ISOLATED PERFUSED RABBIT EAR

Amount of 48/80 Injected (mg. in 0·5 ml.)	Total Histamine in Perfusate (μg.)		
2·0	15·1		
1·0 (3)	13·5	12·1	9·7
0·5 (3)	82·0	18·9	16·3
0·1 (2)	11·0	9·5	
0·05 (2)	11·4	5·8	

The number of experiments is indicated in brackets.

The concentration of histamine in the whole skin of rabbit ear ranged from 16 to 30 μg. per g. in five ears (mean 23.2).

Release of Histamine from Liver.—The liver perfusate, unlike that of the ear, occasionally contained moderate amounts of histamine even when perfused with Locke solution. This varied between 0.01 and 0.07 μg./ml. and usually, though not always, fell to very low concentrations after perfusing for some time. However, transferring the perfusion from Locke's solution to 2% horse serum resulted in a prolonged increase in the output of histamine in six of seven experiments on livers from sensitized animals. The average total output of histamine by antigen in these six experiments was 22.4 μg. (S.D. 14.6). The mean histamine concentration in the perfusate for 20 minutes prior to antigen perfusion was 0.03 μg./ml., and 0.11 in the first 20 minutes during perfusion. A typical result is shown in Fig. 1.

Similar control experiments were done on seven normal preparations. In two of these experiments transferring the perfusion from Locke solution to 2% horse serum produced a release of histamine similar to that occurring in sensitized preparations. Rabbit liver thus occasionally shows a primary release of histamine on perfusion with horse serum. However, unlike the primary histamine release regularly evoked by horse serum from cat skin (Feldberg and Schachter, 1952) or by egg white in the intact cat and rat (Schachter and Talesnik, 1952), it was only occasionally observed in rabbit liver, and never from skin or intestine. Furthermore, it is not due to an occasional general tissue sensitivity, since horse serum perfusion of ears from the same two rabbits failed to release histamine.

Compound 48/80 was injected intraportally in four similar experiments. Injections of 1.0–5.0 mg. were either completely ineffective or produced only

traces of histamine in the effluent, irrespective of whether the perfusion fluid was Locke or horse serum solution. The ineffectiveness of 48/80 contrasts with the ready release of histamine by antigen from this organ of sensitized animals.

Release of Histamine from Intestine.—The basal histamine release from intestine was low, 0.01 μg./ml. or less, and rapidly became undetectable. In three control experiments on intestine from normal rabbits, perfusion with Locke or antigen solution failed to release any histamine or to augment the intestinal movements. In four of five experiments with intestine from sensitized rabbits, transfer of perfusion from Locke to antigen resulted in the release of definite though small amounts of histamine, giving totals of 0.2, 0.5, 1.0, and 3.8 μg. Increased intestinal movements always occurred with this release of histamine.

In three experiments 1 mg. 48/80 (0.05 ml.) was injected intra-arterially. This relatively large amount released no histamine or only traces of it.

Identification of Histamine in Perfusates.—Since Rand and Reid (1952) emphasized that 5-hydroxytryptamine (serotonin, thrombotonin) is present in considerable amounts in rabbit serum and may be readily mistaken for histamine if assayed on guinea-pig intestine, precautions were taken to ensure that the activity of the perfusates was in fact not partially due to the former substance. This was excluded by testing the effect of concentrations of mepyramine (0.4 μg. added to the bath) capable of abolishing the effect of histamine, against contractions elicited by 5-hydroxytryptamine. This had little or no effect on contractions produced by 5-hydroxytryptamine. Sensitive preparations of guinea-pig ileum treated with mepyramine could detect as little as 0.05 μg. and occasionally less of this substance. None the less, the activity of the perfusates was abolished by mepyramine even if two to three times the volume of perfusate assayed for histamine was tested. Therefore, the quantitative assay of the perfusates as histamine could not have included any activity due to 5-hydroxytryptamine.

Experiments with Pontamine Blue Dye.—Six normal rabbits were injected with a 5% solution of pontamine blue in saline by a marginal ear vein (1 ml./kg.). This was followed in 10 minutes by intravenous injection of 48/80 (1 mg./kg.) in two of the rabbits and by horse serum in another two. The remaining two received no further treatment. Thirty minutes after injection of dye all animals were killed by a blow on the head and a skin patch off the flank shaved, depilated, and removed. Three sensitized animals were likewise injected with dye and antigen and treated similarly. The normal animals all showed only faint blueness, and those which received horse serum or 48/80 after the dye showed no greater blueness than those receiving dye alone.

The three sensitized animals which were shocked by antigen, however, all showed markedly increased blueness of the skin, the degree of which appeared to be related to the severity of shock. The increased blueness of the skin was general and became evident in a few minutes. Miles (1953) has also noted widespread blueness of the skin during anaphylactic shock in guinea-pigs. Examination of the viscera revealed a markedly increased staining of the entire gastro-intestinal tract involving all layers of the gut wall. It was difficult to assess the degree of blueness of such normally dark organs as liver and spleen. There was no gross blueness of the lung. It is of interest that the intravenous injection of histamine (100–300 μg./kg.) did not increase permeability to circulating dye. A dose of 300 μg./kg. histamine administered intravenously in 30–60 sec. was found to be fatal in three rabbits under pentobarbitone anaesthesia and in three of four unanaesthetized animals.

A comparison of patches of skin removed from the flanks of normal dyed animals which received horse serum or 48/80 with those of sensitized animals following injection of antigen is shown in Fig. 3.

Plasma Histamine Concentrations in the Normal and Anaphylactic Rabbit.—Blood samples were taken from the femoral artery of normal rabbits

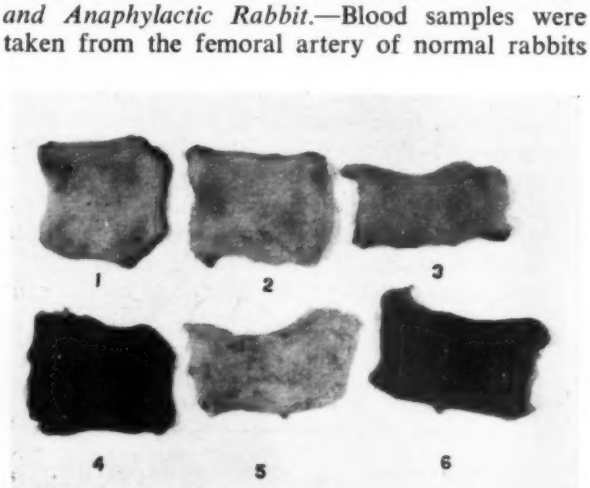

FIG. 3.—Patches of skin removed from the flanks of normal animals (1–3) which received (1) dye alone, (2) dye + 1 ml./kg. horse serum, (3) dye + 1 mg./kg. 48/80. The three animals show no difference in degree of blueness. (4), (5), and (6) are similar patches removed from three dyed animals in shock following 1 mg./kg. horse serum. Tissue (4) is from an animal which underwent severe shock, whereas (5) is from one which had a mild reaction. All substances were administered intravenously.

before and 1½–2 minutes after injections of horse serum (1 ml./kg.) or 48/80 (1 or 2 mg./kg.), and similarly from sensitized animals before and after serum. Centrifugation was immediately carried out and assay of the plasma histamine completed in about 20 minutes after withdrawal of blood. It was observed, as reported for rabbit serum by Rand and Reid (1952), that occasionally the contraction of the guinea-pig ileum produced by rabbit plasma was not entirely due to histamine, since it partially persisted after the preparation was rendered insensitive to histamine by mepyramine. However, mepyramine resistant activity was not detectable in volumes of plasma containing 0.04 μg. histamine or less. Hence the assay of small amounts of plasma (diluted 2 to 10 times if necessary) excluded the effect which was not due to histamine. By testing larger amounts of plasma (0.5–1.0 ml.) it was possible to study changes in the concentration of its mepyramine resistant activity. It is apparent, however, that rabbit plasma which contains several smooth muscle stimulants, and possibly inhibitory substances like adrenaline after shock, may be difficult to assay precisely for histamine. Hence the plasma histamine concentrations must be regarded as approximate.

Horse serum or 48/80 failed to produce significant increases in plasma histamine concentration in normal animals. In previously sensitized animals, however, horse serum produced increased plasma histamine concentrations in eight of nine experiments. The greatest increases occurred in two rabbits which died of shock in less than five minutes. The results were the same with or without siliconed glassware, but the high histamine values occasionally obtained in normal blood did not occur when this added precaution against "spontaneous" histamine release was taken. These results are shown in Table III.

Smooth Muscle Contracting Effect of Rabbit Plasma not Due to Histamine.—The occasional action of plasma (0.5–1.0 ml.) in causing a contraction of the guinea-pig ileum in the presence of mepyramine was also studied. This usually consisted of an immediate rapid contraction followed in about 20 seconds by a superimposed slow one which reached its maximum in approximately 60 seconds. This effect is probably in part due to 5-hydroxytryptamine, as the work of Rand and Reid (1952) on rabbit serum indicates. However, it was only partially reduced by tryptamine desensitization (Gaddum, 1953) of the guinea-pig ileum in the presence of mepyramine, which indicates that it cannot be entirely due to 5-hydroxytryptamine or similar compounds. The slow contracting component persisted after tryptamine desensitization.

Comparison of this activity of plasma obtained from normal animals before and after intravenous injection of horse serum or 48/80 indicated that its concentration paralleled the variations in the

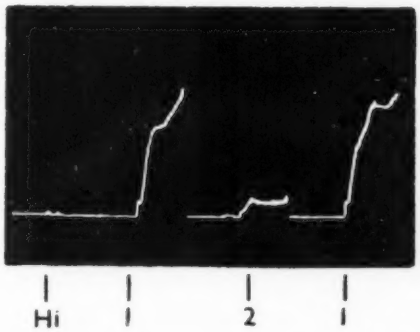

FIG. 4.—Effect of rabbit plasma, obtained before and during anaphylactic shock, on the guinea-pig ileum treated with mepyramine. Contraction time, 60 seconds. Hi, 0.5 μg. histamine. 1, 0.5 ml. plasma before shock. 2, 0.5 ml. plasma 2 minutes after injection of antigen.

TABLE III

PLASMA HISTAMINE CONCENTRATIONS (μg./ml.) IN NORMAL RABBITS BEFORE AND AFTER HORSE SERUM (1 ml./kg.) OR 48/80 (2 mg./kg.) AND IN SENSITIZED RABBITS BEFORE AND 1.5–2.0 MINUTES AFTER THE INTRAVENOUS INJECTION OF HORSE SERUM (1 ml./kg.)

Normal Rabbits						Sensitized Rabbits			
48/80			Horse Serum			Horse Serum			
Exp. No.	Before Injection	After Injection	Exp. No.	Before Injection	After Injection	Exp. No.	Before Injection	After Injection	Severity of Shock (Effect on Arterial B.P.)
1†	0·05	0·05	1	0·05	0·08	1	0·12	0·25	Not recorded
2	0·04	0·05	2	0·34	0·26	2	0·12	0·75	" "
3	0·20	0·08	3	0·16	0·33	3	0·20	1·80	Very severe; dead in 5 min.
4*	0·05	0·05	4	0·05	0·03	4	0·32	1·20	" " " " "
			5	0·02	0·01	5	0·34	1·0	Moderate; considerable recovery in 20 min.
			6*	0·13	0·17	6	0·60	0·50	
			7*	0·06	0·07	7*	0·12	0·60	Severe; dead in 35 min. " "
						8*	0·08	0·20	Severe; dead in 60 min.
						9*	0·10	0·22	Moderate; considerable recovery in 20 min.

* All glassware siliconed. † 1 mg./kg. 48/80 injected.

histamine concentration. In sensitized animals, however, there was regularly a reduction of this activity 1½–2 minutes after intravenous injection of antigen, even when elevation of the plasma histamine concentration occurred. Fig. 4 illustrates a typical experiment; it shows the reduced contraction of the guinea-pig ileum in the presence of mepyramine, caused by rabbit plasma which was obtained during severe anaphylactic shock, when compared with a control sample of plasma from the same animal. In this experiment the plasma histamine concentration had risen at the same time from 0.34 to 1.0 μg./ml.

Discussion

The demonstration in the present experiments of the release of considerable amounts of histamine from isolated rabbit organs during the antigen-antibody reaction indicates that the release of histamine contributes to the toxicology of the anaphylactic reaction in this species. Its role in rabbit anaphylaxis is, therefore, qualitatively similar to that in the guinea-pig and dog. It is furthermore worth noting that the effect of the local release of histamine within specific organs may be more drastic than that due to histamine injected into the blood stream. For example, in the unanaesthetized dog the response to histamine release from skin is accompanied by severe cutaneous reactions, such as erythema, pruritus, and oedema, although no such effects occur when the amounts of histamine released by the whole animal are administered systemically (Paton and Schachter, 1951). Also, in the present experiments the intravenous injection of histamine in amounts up to the lethal dose failed to increase capillary permeability as evidenced by the degree of staining of tissues in the presence of circulating pontamine blue. However, even in non-lethal anaphylaxis there was a marked increase in blueness of the skin and gastro-intestinal tract.

The decrease in histamine concentration of whole blood which occurs in anaphylaxis (Rose *et al.*, 1939, 1941), despite its release from tissues, may be associated with the simultaneous trapping of platelet thrombi in various organs which occurs in shock (Fidler and Waters, 1946). Since the amount of histamine in rabbit platelets accounts for most of the blood histamine in this species (see Code, 1952), such a removal of platelets from circulating blood might greatly reduce the whole blood histamine concentration. Various other mechanisms, for example, the increased serum histaminase activity which occurs in anaphylactic shock (Rose and Leger, 1952), may also be involved. It is of interest in this regard that injection of less effective histamine liberators such as bile salt and neoarsphenamine produces a reduction in plasma histamine concentration in the cat (Schachter, 1952). The significant fact is, however, that histamine is released from a variety of rabbit tissues during the antigen-antibody reaction. Also, in the present experiments, elevation of the plasma histamine concentration was demonstrated in most instances of severe anaphylactic reactions.

The present experiments do not permit a precise assessment of the contribution of histamine to the lethal anaphylactic reaction, but the total amounts released from isolated organs by antigen indicate that enough may be released in the intact animal to produce severe or fatal reactions if it were administered by the intravenous route. For example, the average amount released from the skin of one ear alone was 34.6 μg. Perfusion experiments also demonstrated considerable release from liver (mean 22.4 μg.) and small but definite amounts from intestine. Also, substantial amounts of histamine are known to be released from the formed elements of rabbit blood *in vitro*, resulting in a mean increase of 0.8 μg./ml. plasma (calculation based on results of Carryer and Code, 1950). Since the experiments with pontamine blue support the probability that the anaphylactic reaction occurs in the skin generally as well as in the entire gastro-intestinal tract, it seems likely that in severe reactions the total amount of histamine released approaches the demonstrated lethal intravenous dose of histamine—viz., 300 μg./kg.

Recent experiments have, under various conditions, demonstrated the release of smooth muscle stimulating substances other than histamine during the antigen-antibody reaction (see Introduction). Our experiments have failed to detect the release or formation of non-histamine smooth muscle stimulants in perfusates from perfused skin, liver, or intestine as tested on the guinea-pig ileum. The release or formation of significant amounts of such substances must, therefore, either require the presence of blood or originate therefrom. However, the non-histamine smooth muscle contracting activity of rabbit plasma was regularly decreased at the height of shock in the intact animal even when elevations of plasma histamine concentrations occurred. Although the present results emphasize the importance of the release of histamine in rabbit anaphylaxis, it is quite possible that other released or formed substances contribute to the total reaction, as for example the release of heparin in canine anaphylaxis (Jaques and Waters, 1941) accounts for the incoagulability of the blood.

The fact that compound 48/80 failed to release histamine from perfused rabbit liver, whereas antigen readily did so, may indicate a significant difference in the mechanism of histamine release by these two agents. It would be of interest to extend this comparison to the dog, where the liver plays such a prominent role in anaphylactic shock.

Summary

1. In normal rabbits, horse serum failed to release histamine from perfused skin and intestine, but occasionally did so from liver. It had no effect on the arterial blood pressure, and failed to alter permeability to circulating pontamine blue dye or to increase the concentration of plasma histamine.

2. In sensitized rabbits horse serum released considerable amounts of histamine from perfused organs. The greatest release was obtained from the skin of the isolated ear, moderate release from liver, and least from small intestine. Increased permeability to circulating dye occurred in shocked animals as evidenced by markedly increased blueness of the skin and gastro-intestinal tract, and increased plasma histamine concentrations were demonstrated in most instances of fatal or severe shock. These observations indicate that histamine release is a significant factor in the toxicology of the acute anaphylactic reaction in the rabbit.

3. The amount of histamine released from the skin of the perfused ear during the antigen-antibody reaction, plus the amount subsequently remaining, corresponded to the total amount of histamine extractable from the untreated ear. It is concluded, therefore, that the histamine released during this reaction is not formed, but comes from a pre-existing store.

4. Rabbit plasma occasionally exhibited a non-histamine smooth muscle contracting activity which was only partially due to 5-hydroxytryptamine, since a slow contraction of the guinea-pig ileum persisted after tryptamine desensitization. This activity, when present, was reduced in samples of plasma taken two minutes after anaphylactic shock.

5. Antigen was more effective in releasing histamine from perfused rabbit organs than the histamine releasing substance, 48/80. This was particularly true for the perfused liver, where the injection of large amounts of 48/80 failed to release histamine.

6. Assay of perfusates from skin, liver, and intestine, tested on the guinea-pig ileum, failed to demonstrate the presence of smooth muscle stimulating substances other than histamine after infusion of antigen or injection of 48/80.

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THE TRYPANOCIDAL ACTIVITY OF DITHIOBIURET DERIVATIVES

BY

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(RECEIVED JUNE 12, 1953)

During the routine screening of various types of compounds for chemotherapeutic activity, trypanocidal activity was noted in several dithiobiuret derivatives. As a consequence, a more detailed study of compounds of this type was carried out.

The chemistry of the compounds mentioned in this paper will be reported elsewhere by Fairfull and Peak.

MATERIALS AND METHODS

Most of the compounds had a very low solubility in water, and only for those marked with an asterisk in the Tables was this greater than 1 in 500. The drugs were therefore used in aqueous suspension, with Dispersol LN as a wetting and dispersing agent. They were given subcutaneously to mice showing a moderate degree of parasitaemia following inoculation with diluted infected mouse blood. Many of the compounds were tested against several trypanosome species—*Trypanosoma rhodesiense, T. gambiense, T. brucei, T. congolense,* and *T. cruzi*—but none showed appreciable trypanocidal activity against any species other than *T. congolense.* In the Tables, therefore, the results given are for activity against the F.N. strain of *T. congolense* only. The largest dose given was usually a high fraction of the acute subcutaneous LD50, but the maximum dose used was 500 mg./kg. Doses were given on one occasion to one series of mice, and repeated daily for five days in a second series of animals. In general the drugs were more active when the same total amount was given in repeated doses than in a single dose. The CD50 has been calculated on the basis of removal of parasites from the peripheral blood for one month.

RESULTS

1-Alkyldithiobiurets, etc. (Table I).—Dithiobiuret itself, RD2491, is known to be a compound of fairly high toxicity. Nevertheless, repeated doses can cure mice infected with *T. congolense.* 1-Methyl substitution (RD1658) increased activity, while the 1-ethyl compound was less toxic than, and approximately as active as, unsubstituted dithiobiuret. However, lengthening the alkyl chain increased toxicity and decreased activity.

TABLE I

THE TRYPANOCIDAL ACTIVITY OF 1-ALKYLDITHIOBIURETS, ETC.

R—NH.C(=S).NH.C(=S).NH$_2$

Reference No.	R =	Acute LD50 mg./kg. Subcutaneously	Activity against *T. congolense*
RD2491	H—	*	Delayed death at 100 mg./kg. s.c.×1. CD50 *ca.* 40 mg./kg. s.c.×5
RD1658	Methyl	140	Delayed death at 80 mg./kg. s.c.×1. CD50 *ca.* 20 mg./kg. s.c.×5
RD1659	Ethyl	*ca.* 1,000	CD50 *ca.* 300 mg./kg. s.c.×1. *ca.* 40 mg./kg. s.c.×5
RD1668	*n*-Butyl	*ca.* 200	Delayed death at 100 mg./kg. s.c.×1. CD50 *ca.* 80 mg./kg. s.c.×5

1-Aryldithiobiurets.—Many of the compounds listed in Table II had considerable activity when repeated doses were given, and the toxicity generally was low. The soluble compounds (marked with an asterisk) were usually more toxic than the practically insoluble derivatives, so that the apparent low toxicity of, for example, RD1501 might result from poor absorption. Nevertheless, even compounds of low solubility, such as RD1501, had quite marked trypanocidal activity. Of the members of the homologous series of 1-*p*-alkylphenyldithiobiurets, the methyl compound, RD1548, was the most potent. In the alkoxyphenyl- series the *para*- substituted compound RD1145 was more effective than the *meta*- or the *ortho*- analogues when five doses were given; but, rather curiously, it was the least effective of the three when only a single dose was administered. The ethoxy- and *n*-butoxy-derivatives were less potent than the methoxy-compound. Of other *p*-phenyl substituents tried, only the *p*-chloro-compound had retained appreciable activity.

None of the naphthyl-, pyridyl-, *iso*quinolyl-, or quinolyl-derivatives tried had more than slight activity.

TABLE II

THE TRYPANOCIDAL ACTIVITY OF 1-ARYL-DITHIOBIURETS

R—NH.C(=S).NH.C(=S).NH$_2$

Reference No.	R =	Acute LD50 mg./kg. Subcutaneously	Activity against *T. congolense*
RD1501	Phenyl	4,500	CD50 *ca.* 150 mg./kg. s.c.×1; *ca.* 35 mg./kg. s.c.×5
RD1548	*p*-Tolyl	>5,000	CD50 *ca.* 180 mg./kg. s.c.×1; *ca.* 25 mg./kg. s.c.×5
RD2138	*p*-*n*-Propylphenyl	>5,000	Delayed death at 200 mg./kg. s.c.×1; CD50 *ca.* 70 mg./kg. s.c.×5
RD2286	*p*-*n*-Butylphenyl	>3,200	CD50 *ca.* 400 mg./kg. s.c.×1; *ca.* 200 mg./kg. s.c.×5
RD1519	*o*-Methoxyphenyl	*ca.* 3,200	CD50 *ca.* 180 mg./kg. s.c.×1; *ca.* 80 mg./kg. s.c.×5
RD1549	*m*-Methoxyphenyl	>5,000	CD50 *ca.* 200 mg./kg. s.c.×1; *ca.* 50 mg./kg. s.c.×5
RD1145	*p*-Methoxyphenyl	*ca.* 500 i.p.	Delayed death at 200 mg./kg. s.c.×1; CD50 *ca.* 40 mg./kg. s.c.×5
RD1524	*p*-Ethoxyphenyl	>5,000	Delayed death at 500 mg./kg. s.c.×1; CD50 *ca.* 115 mg./kg. s.c.×5
RD1547	*p*-*n*-Butoxyphenyl	>5,000	Delayed death at 500 mg./kg. s.c.×1; CD50 *ca.* 150 mg./kg. s.c.×5
RD1518	*p*-Chlorophenyl	2,000	CD50 *ca.* 150 mg./kg. s.c.×1; *ca.* 45 mg./kg. s.c.×5
RD1546	*p*-Ethylsulphonylphenyl	>5,000	Delayed death at 500 mg./kg. s.c.×1; CD50 *ca.* 350 mg./kg. s.c.×5
RD1657	*p*-Carboxyphenyl	*ca.* 1,000	Delayed death at 400 mg./kg. s.c.×1; or 300 mg./kg. s.c.×5
RD1656	*p*-Carbethoxyphenyl	*ca.* 4,000	Delayed death at 500 mg./kg. s.c.×1; or 500 mg./kg. s.c.×5
RD1609	*p*-Dimethylaminophenyl	>5,000	De'ayed death at 500 mg./kg. s.c.×1; or 500 mg./kg. s.c.×5
RD1898	*p*-Dimethylaminophenyl (methochloride) *	*ca.* 400	Inactive at 100 mg./kg. s.c.×1; or 100 mg./kg. s.c.×5
RD2103	*p*-Acetamidophenyl	>5,000	Inactive at 400 mg./kg. s.c.×1; CD50 *ca.* 250 mg./kg. s.c.×5
RD1573	β-Naphthyl	200	Inactive at 100 mg./kg. s.c.×1; or 50 mg./kg. s.c.×5
RD1666	α-Naphthyl	>5,000	Delayed death at 500 mg./kg. s.c.×1; CD50 *ca.* 300 mg./kg. s.c.×5
RD1844	2-Pyridyl	*ca.* 4,000	Delayed death at 400 mg./kg. s.c.×1; or 400 mg./kg. s.c.×5
RD1951	3-Pyridyl	*ca.* 800	Inactive at 400 mg./kg. s.c.×1; delayed death at 400 mg./kg. s.c.×5
RD2039	3-Pyridyl (methochloride) *	280	Inactive at 50 mg./kg. s.c.×1; or 50 mg./kg. s.c.×5
RD2040	4-*Iso*quinolyl	>5,000	Delayed death at 400 mg./kg. s.c.×1; or 400 mg./kg. s.c.×5
RD2137	4-*Iso*quinolyl (methochloride) *	*ca.* 2,000	Inactive at 400 mg./kg. s.c.×1; or 400 mg./kg. s.c.×5

TABLE II (*contd.*)

Reference No.	R =	Acute LD50 mg./kg. Subcutaneously	Activity against *T. congolense*
RD2284	5-Quinolyl	*ca.* 1,000	Delayed death at 500 mg./kg. s.c.×1; CD50 *ca.* 100 mg./kg. s.c.×5
RD1985	6-Quinolyl	*ca.* 2,000	Inactive at 500 mg./kg. s.c.×1; delayed death at 500 mg./kg. s.c.×5
RD1986	6-Quinolyl (methochloride) *	>5,000	Inactive at 500 mg./kg. s.c.×1; delayed death at 250 mg./kg. s.c.×5 (toxic at 500 mg./kg. s.c.×5)

TABLE III

THE TRYPANOCIDAL ACTIVITY OF :-ARYL-1-ALKYL-DITHIOBIURETS

C_6H_5—N(R)—C(=S)—NH—C(=S).NH$_2$

Reference No.	R	Acute LD50 mg./kg. Subcutaneously	Activity against *T. congolense*
RD1660	Methyl	*ca.* 1,000	CD50 *ca.* 200 mg./kg. s.c.×1; *ca.* 10 mg./kg. s.c.×5
RD1704	Ethyl	1,400	CD50 *ca.* 90 mg./kg. s.c.×1; *ca.* 17 mg./kg. s.c.×5
RD1823	*n*-Propyl	*ca.* 5,000	CD50 *ca.* 100 mg./kg. s.c.×1; *ca.* 25 mg./kg. s.c.×5
RD1705	*n*-Butyl	>4,000	CD50 *ca.* 150 mg./kg. s.c.×1; *ca.* 40 mg./kg. s.c.×5

TABLE IV

THE TRYPANOCIDAL ACTIVITY OF 1,5-DISUBSTITUTED-DITHIOBIURETS

R^1—NH.C(=S).NH.C(=S).NH—R^2

Reference No.	R^1	R^2	Acute LD50 mg./kg. Subcutaneously	Activity against *T. congolense*
RD1706	Phenyl	Ethyl	*ca.* 5,000	CD50 *ca.* 300 mg./kg. s.c.×1; *ca.* 120 mg./kg. s.c.×5
RD1707	Phenyl	*n*-Butyl	*ca.* 1,800	Delayed death at 500 mg./kg. s.c.×1; CD50 *ca.* 250 mg./kg. s.c.×5
RD1737	Phenyl	Phenyl	>3,800	Inactive at 500 mg./kg. s.c.×1; delayed death at 400 mg./kg. s.c.×5
RD1750	Phenyl	*p*-Methoxyphenyl	>5,000	Delayed death at 500 mg./kg. s.c.×1; CD50 *ca.* 300 mg./kg. s.c.×5
RD1749	*p*-Methoxyphenyl	*p*-Methoxyphenyl	*ca.* 1,500	Delayed death at 400 mg./kg. s.c.×1; CD50 *ca.* 200 mg./kg. s.c.×5
RD2276	Phenyl	Diphenylmethyl	>3,200	Inactive at 500 mg./kg. s.c.×1; delayed death at 500 mg./kg. s.c.×5

1-Alkyl-1-aryl-dithiobiurets (Table III).—These compounds appeared to show considerable promise, and the 1-methyl-1-phenyl-compound (RD1660) was the most effective compound of the whole series tested. Again, however, repeated doses were necessary.

1, 5-Disubstituted-dithiobiurets.—Only slight activity, if any, was shown by this series of compounds (Table IV).

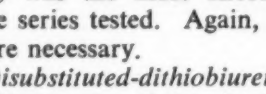
1 : 1 : 5-Trisubstituted-dithiobiurets.—Only two such compounds were tested. Both had appreciable activity, but they were inferior to the 1-alkyl-l-aryl-dithiobiurets (Table V).

Bis(1-dithiobiurets).—In view of the activity of 1-aryldithiobiurets it was thought interesting to try bis(1-dithiobiurets). However, none of the three compounds tested showed any detectable trypanocidal activity (Table VI).

Iso-*dithiobiuret Derivatives* (Table VII).—None of the compounds tested had more than a trace of activity.

TABLE V

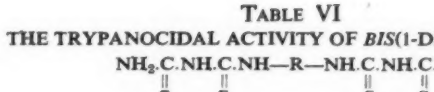
THE TRYPANOCIDAL ACTIVITY OF 1:1:5-TRISUBSTITUTED-DITHIOBIURETS



Reference No.	R	Acute LD50 mg./kg. Subcutaneously	Activity against *T. congolense*
RD2102	Methyl	*ca.* 5,000	CD50 *ca.* 250 mg./kg. s.c.×1; CD50 *ca.* 30 mg./kg. s.c.×5
RD2217	*n*-Propyl	>4,000	CD50 *ca.* 300 mg./kg. s.c.×1; CD50 *ca.* 70 mg./kg. s.c.×5

TABLE VI

THE TRYPANOCIDAL ACTIVITY OF *BIS*(1-DITHIOBIURETS)



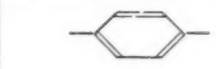
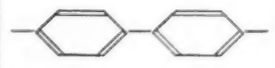
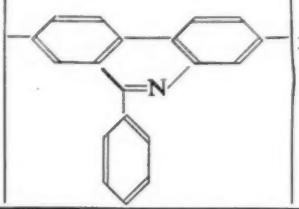
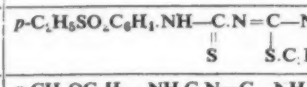
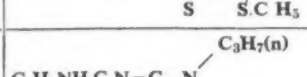
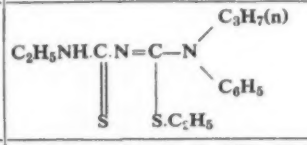
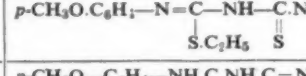
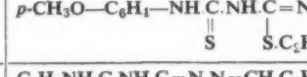
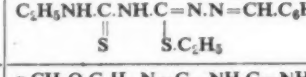
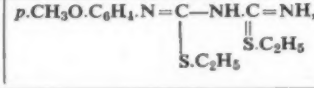
Reference No.	R=	Acute LD50 mg./kg. Subcutaneously	Activity against *T. congolense*
RD2285		>3,200	Inactive at 500 mg./kg. s.c.×1; or 500 mg./kg. s.c.×5
RD2038		>5,000	Inactive at 400 mg./kg. s.c.×1; or 400 mg./kg. s.c.×5
RD2072		>5,000	Inactive at 200 mg./kg. s.c.×1; or 200 mg./kg. s.c.×5

TABLE VII

THE TRYPANOCIDAL ACTIVITY OF *ISO*-DITHIOBIURET DERIVATIVES

Reference No.	Structure	Acute LD50 mg./kg. Subcutaneously	Activity against *T. congolense*
RD1477		>5,000	Inactive at 500 mg./kg. s.c.×1; delayed death at 500 mg./kg. s.c.×5
RD1146		750 (i.p.)	Inactive at 250 mg./kg. s.c.×1; or 250 mg./kg. s.c.×5
RD2109		*ca.* 2,000	Inactive at 400 mg./kg. s.c.×1; or 200 mg./kg. s.c.×5
RD2153			Inactive at 500 mg./kg. s.c.×1; or 500 mg./kg. s.c.×5
RD1249		*ca.* 2,000	Inactive at 500 mg./kg. s.c.×1; or 500 mg./kg. s.c.×5
RD1353		*ca.* 1,500	Inactive at 500 mg./kg. s.c.×1; delayed death at 500 mg./kg. s.c.×5
RD1248		>800	Inactive at 300 mg./kg. s.c.×1; or 300 mg./kg. s.c.×5

Discussion

From the results obtained it is clear that compounds of the dithiobiuret type may possess considerable activity against *Trypanosoma congolense* infections in mice, and that even dithiobiuret itself can cure the infection when five daily doses are given. This need for repeated doses has been noted with practically all the compounds tested, and is rather unexpected. It might be assumed that, if a single massive dose of a compound of low solubility were given subcutaneously, there would be slow absorption and a maintained blood level of the drug. This, apparently, is not so in the dithiobiuret series, and it might be interesting to speculate on the fate of single large doses. In none of the mice killed and examined was there enough encapsulation fully to explain the results, though in some mice residual masses of the drug could be found several days after administration. Most of the compounds had low acute toxicity by the subcutaneous route, and several had a high ratio between acute LD50 and CD50 (five doses); thus RD1660 (Table III) had LD50 approximately 1,000 mg./kg., whereas the CD50 was of the order of 10 mg./kg. given on five successive days. The corresponding figures for RD1823 (Table III) are 5,000 and 25. Such figures compare favourably with all but the most potent of drugs used against *T. congolense*. The need for repeated dosing is, however, an obstacle which might handicap field use of these compounds in cattle. The unavoidable inaccuracies in dosing with suspensions of insoluble drugs would also militate against their use. Nevertheless, it is hoped that some of the more active compounds described will be given a field trial in *T. congolense* infections.

Summary

1. Dithiobiuret and several derivatives have been shown to possess activity against *T. congolense* when given subcutaneously to infected mice.

2. 1-Alkyl-l-aryl dithiobiurets were the most potent derivatives tested.

3. Repeated doses appeared to be necessary for the full therapeutic effect to be shown.

4. The ratio between acute LD50 and CD50 was high for many of the compounds.

5. Because of the need for repeated doses it is concluded that field use of the compounds might be difficult.

I am grateful to Mrs. B. Goodliffe and to Miss H. Riley for much technical assistance, and to Drs. A. E. Fairfull and D. A. Peak, who supplied the compounds tested.

Brit. J. Pharmacol. (1953), **8**, 424.

THE MODE OF ACTION OF PROGUANIL AND RELATED ANTIMALARIAL DRUGS

BY

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(RECEIVED JUNE 18, 1953)

During recent years considerable interest has been aroused by the ability of a number of substances to antagonize pteroylglutamic acid in both microbial and mammalian systems. Discussing the antagonistic effects of substituted 2:4-diaminopyrimidines, Falco, Hitchings, Russell, and VanderWerff (1949) pointed out that these substances showed a formal resemblance to proguanil ("Paludrine"), and might therefore be expected to have antimalarial activity also. They suggested, too, that by virtue of this similarity proguanil should display pterolyglutamic acid antagonism in their bacterial cultures. Development of the idea that 2:4-diaminopyrimidines might possess antimalarial activity led to the synthesis of pyrimethamine (2:4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine; "Daraprim"), one of the most active compounds of the series, as an antimalarial drug.

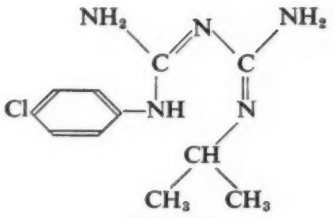
Soon after the introduction of proguanil as an antimalarial, Hawking (1947) and Hawking and Perry (1948) produced evidence to suggest that the drug itself did not possess antimalarial activity, but that it was metabolized in the body to an active compound. One such active metabolite was isolated from the urine of proguanil-treated rabbits by Carrington, Crowther, Davey, Levi, and Rose (1951), and shown to be 2:4-diamino-1-*p*-chlorophenyl-1:6-dihydro-6:6-dimethyl-1 : 3 : 5-triazine, which bears a marked structural resemblance to pyrimethamine (see formulae).

The present study was undertaken to obtain further information on the bearing of the metabolite on the antimalarial action of proguanil, and on the correlation, if any, between antimalarial activity and interference by substances active in the pteroylglutamic acid system. Other drugs, related to proguanil and its metabolite, have been used to obtain additional information. The structural formulae of the substances are given on the next page.

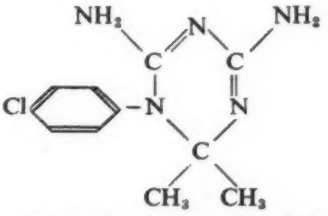
METHODS

L. casei was cultivated in a medium containing a charcoal-treated tryptic digest of casein, supplemented with inorganic salts, glucose, and growth factors; 1 l. of the final growth medium contained the equivalent of 25 g. casein, $MgSO_4.7H_2O$ 800 mg., $MnSO_4.4H_2O$ 160 mg., $FeSO_4.(NH_4)_2SO_4.6H_2O$ 56 mg., glucose 10 g., L-cysteine 100 mg., adenine 7 mg., guanine 2 mg., thiamin, nicotinic acid, Ca pantothenate and pyridoxin 1 mg. each, riboflavine 0.4 mg., and biotin 1 μg. The medium was prepared in a concentrated form, and distributed in 3.5-ml. amounts in $5 \times \frac{5}{8}$-in. tubes plugged with absorbent cotton-wool covered with surgical gauze. 0.5 ml. pteroylglutamic acid (PtG) was added to each tube to give $10^{\frac{1}{2}}$- (i.e., 3.16-) fold final dilutions over the range 10^{-5} to 10^{-11} M (i.e., 4.4 to 0.0000044 μg./ml.). The tubes were autoclaved for 10 min. at 10 lb./sq. in., cooled, and 0.5 ml. drug solution, sterilized by Seitz filtration, was added. The tubes were inoculated with 0.5 ml. of a dilute suspension of bacteria and incubated in an atmosphere of 5% CO_2–95% N_2 (v/v) for 40 hours at 37° C. Growth was measured using the Hilger Spekker absorptiometer and a 0.5 cm. cell. Curves were plotted correlating the growth obtained with $\log_{10}$ (PtG concentration) in the absence and in the presence of variable quantities of drug. Two tubes were used for each concentration of PtG, so that each growth curve was based on 28 tubes. Curves were plotted representing growth in the presence of different fixed concentrations of drug; the drugs were used in $10^{\frac{1}{2}}$-fold dilutions, five different concentrations being examined with one control in any particular experiment.

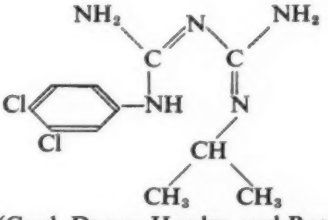
A normal strain of *Plasmodium gallinaceum* was used, and also a strain made resistant to 2 mg. proguanil (all doses are expressed as mg. drug given orally b.i.d. × $3\frac{1}{2}$ to a 50-g. chick). This resistant strain had been passaged in the presence of proguanil over a period of $10\frac{1}{2}$ months. The sensitivity of these two strains to the various drugs under test was determined by the standard therapeutic test of Davey (1946); the minimum effective dose (MED) was taken to be the lowest dose which would reduce the parasite count below 10 per 500 erythrocytes (count in untreated controls 300–400 parasites per 500 erythrocytes).



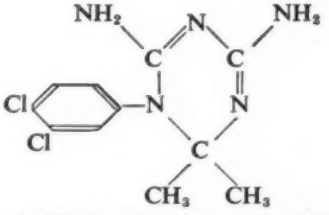
Proguanil



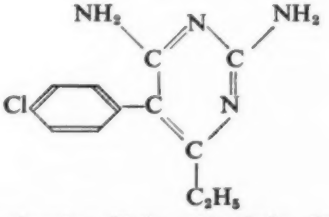
10,580 (Carrington *et al.*, 1951)



5943 (Curd, Davey, Hendry, and Rose, 1950)



10,732 (Carrington *et al.*, 1951)



Pyrimethamine (Falco, Goodwin, Hitchings, Rollo, and Russell, 1951)

Previous studies of the effects of antimalarial drugs on the erythrocytic stages of malaria parasites *in vitro* have been done using the rocker-dilution technique of Geiman, Anfinsen, McKee, Ormsbee, and Ball (1946) or a modified Bass and Johns method (e.g., Hawking, 1947). In the present work it was found convenient to use a method developed by my colleague, Dr. D. G. Davey. The method has not been described before and it is therefore given here in detail. The culture tubes, modified from a design due to Lindbergh (Parker, 1938), are illustrated diagrammatically in Fig. 1. The tubes were filled with

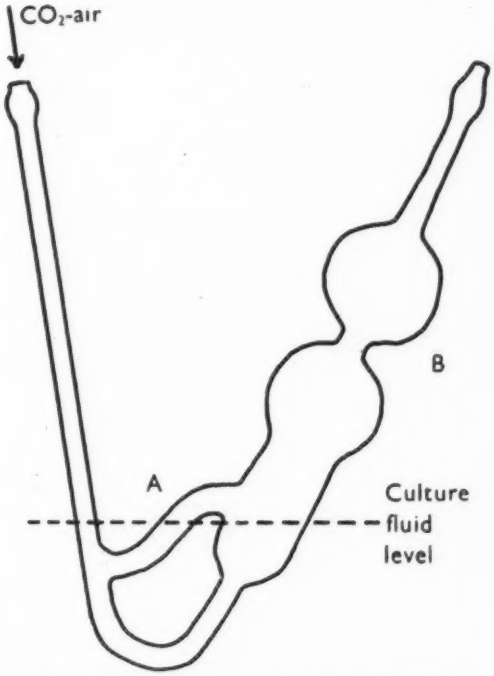


FIG. 1.—Culture tube for malaria parasites. $\times \frac{1}{4}$.

erythrocytic culture to the level indicated in the figure (with the particular tubes in use, this required 4 ml. of culture). By blowing a stream of gas mixture down the inlet tube, small slugs of culture were carried up the gas lift A. In this way the culture was kept continually mixed, and in equilibrium with the gas phase. During aeration the serum in the culture medium caused a certain amount of frothing; this froth was broken down in the bulbs B. For culture experiments, 12 of the tubes were connected in series with rubber tubing. The whole assembly was sterilized by autoclaving, and culture components were introduced by means of sterile syringes through the rubber connecting tubes (sterilized locally by means of 70% ethanol). The gas mixture of 5% CO_2–95% air (v/v) was sterilized by passage through a long column of cotton-wool, and was saturated with water vapour by bubbling through two wash-bottles of water. Cultures were incubated at 38° C. for 24 or 40 hours.

The cultures contained approximately 1.2×10^6 erythrocytes per ml., suspended in a liquid phase consisting of equal parts of chicken serum and a solution containing 0.111 M NaCl, 0.005 M KCl, 0.002 M $MgCl_2$, 0.020 M $NaHCO_3$, 0.002 M phosphate buffer (*p*H 7.4), and 0.020 M glucose, which, when used in conjunction with a gas phase containing 5% CO_2, had a *p*H of 7.4. Blood was removed under aseptic

conditions from infected chickens, and was diluted with clean blood to give a parasitaemia of approximately 30% ; heparin was used as anticoagulant. This infected blood (1 vol.) was added to a mixture of sterile chicken serum and Ringer-glucose-bicarbonate solution (6 vol.), and 3.0 ml. of the resulting mixture was introduced into each culture tube. 1 ml. of Ringer-glucose-bicarbonate solution containing any drugs or growth factors to be studied was then added to each culture. All drug solutions and sera were sterilized by Seitz filtration, and the sterility of the cultures was checked by inoculation of a blood plate. It was found unnecessary to add antibiotics to the cultures to ensure sterility, as has been done by some previous workers.

0.2 ml. of culture was inoculated into 2 or 3 clean chickens (50 g.) before and after incubation, and blood smears taken from these chickens at 24-hr. intervals were examined for the presence of parasites. The infection was regarded as patent when at least three parasites were found in 100 oil-immersion microscope fields examined, and the course of infection was then followed for 3 or 4 days. By comparing this course of infection with that in the controls—provided by material from the cultures before *in vitro* incubation, or from cultures incubated in the absence of drug—it was possible to make an estimate of the prepatent-period delay, and hence of the survival of the parasites following incubation *in vitro* and after incubation in the presence of drug. It was found that an increase in prepatent period of 1.2 days (range 1.1 to 1.6 days in 7 experiments) corresponded to a mortality (or reduction in inoculum) of 90%, and an increase in prepatent period of 2.4 days (range 2.1 to 3.0 days in 7 experiments) to a 99% mortality. Taylor, Greenberg, Josephson, and Ray (1951) found a prepatent-period increase of 1.3 days corresponded to a mortality of 90%. The cultures at the end of the experiments were gently centrifuged, and smears made from the deposit were stained with Giemsa and examined.

Results

The Effect of Proguanil and Related Substances on the Growth-Promoting Properties of Pteroylglutamic Acid for Lactobacillus casei

L. casei grown in the standard PtG assay medium gave half-maximum growth with 2.25×10^{-10} M PtG, while maximum growth was obtained with approximately ten times this concentration of PtG. In the presence of drug concentrations of the order of 10^{-4} M or greater, no bacterial growth took place, no matter how much PtG was added ; in the presence of low drug concentrations (about 10^{-6} M), no inhibition of bacterial growth was observed. With the compounds 10,580, 10,732, and pyrimethamine it was found that intermediate concentrations of drug produced an inhibition of bacterial growth which could be completely reversed by the addition of suitable amounts of PtG. Curves representing bacterial growth in the presence of a fixed concentration of drug were similar in shape and parallel to the control curve, being displaced towards higher PtG concentrations. This competitive inhibition was usually observed over approximately a hundredfold range of drug concentration. Drug activity was expressed in terms of the inhibition index, which represents the ratio of concentrations of drug and growth factor at which their effects are just counterbalanced (based on a 50% reduction of growth). In contrast to the results obtained with pyrimethamine and the two triazines it was found that proguanil and 5943 exerted an " all or none " effect on bacterial growth ; in no experiment was it observed that either of these compounds produced a toxicity which was in any way reversible by added PtG. The results of these experiments are summarized in Table I.

TABLE I

PtG AND THYMINE ANTAGONISM WITH *LACTOBACILLUS CASEI*

Drug	Drug Concentrations (M) Showing			Inhibition Index*	
	Full Growth	Competitive Inhibition	Complete Inhibition	PtG	Thymine
Proguanil	$3{\cdot}16 \times 10^{-4}$	—	10^{-3}	—	—
10,580 ..	$3{\cdot}16 \times 10^{-7}$	10^{-6} to 10^{-4}	10^{-3}	6,500	2·0
5943 ..	$3{\cdot}16 \times 10^{-5}$	—	10^{-4}	—	—
10,732 ..	$3{\cdot}16 \times 10^{-7}$	10^{-6} to $3{\cdot}16 \times 10^{-5}$	$3{\cdot}16 \times 10^{-4}$	6,500	0·9
Pyrimethamine	10^{-7}	$3{\cdot}16 \times 10^{-7}$ to $3{\cdot}16 \times 10^{-5}$	10^{-4}	1,800	0·1

* $\dfrac{\text{Concentration of drug}}{\text{Concentration of growth factor}}$

Thymine Antagonism with Lactobacillus casei

L. casei is able to grow to a limited extent in the presence of thymine and adenine, but in the absence of PtG. Growth curves were obtained for bacteria cultivated in the presence of 10^{-5} M guanine, 5×10^{-5} M adenine, and thymine in 10^{1}-fold dilutions over the range 3.16×10^{-3} to 3.16×10^{-5} M. It was found that half-maximum growth was obtained with about 10^{-4} M thymine, and maximum growth with about ten times this concentration ; in the presence of thymine, maximum growth was about half that obtained in the presence of optimal amounts of PtG. In the thymine systems, competitive inhibition or irreversible toxicity was observed at the same drug concentrations found effective in the PtG systems. Inhibition indices were calculated as before ; the approximate values are given in Table I. As in the

PtG systems, proguanil and 5943 were found to be either non-toxic or to produce a toxicity not reversed by the addition of more growth factor; there was again no evidence of competitive inhibition with these two drugs.

The Effect of Proguanil and Related Compounds on Plasmodium gallinaceum

Drug Sensitivity in vivo.—When assayed against the normal strain of *P. gallinaceum in vivo* the metabolite 10,580 was found to have ten times the activity, while 10,732 showed a hundred times the activity of proguanil; 5943 showed five times the activity, and pyrimethamine fifty times the activity of proguanil. With the resistant strain, however, fully effective doses were not tolerated by chicks. Thus, 10,732 gave an MED of 3 mg., while 5943 and 10,580 showed slight activity over the ranges 2–4 mg. and 5–7 mg. respectively. With the maximum tolerated doses (over the 5-day period of the test), proguanil at 3 mg. and pyrimethamine at 2.5 mg. were completely inactive. These results are summarized in Table II.

TABLE II

IN VIVO DRUG SENSITIVITY OF *P. GALLINACEUM*

(Figures represent the MED for proguanil, 5943, 10,580, and 10,732 as the hydrochlorides, or pyrimethamine as the free base)

	Normal Strain	Resistant Strain
Proguanil	0·25	>3
10,580	0·025	>7
5943	0·05	>4
10,732	0·0025	3
Pyrimethamine	0·005	>2·5

Morphological Effects Produced by Proguanil in vivo.—A number of 6-day-old chicks were inoculated with 50,000,000 parasites, and blood smears taken twice a day. When the parasitaemia reached a level of about 6% twice-daily dosing with 1 mg. proguanil was started. Initially, parasites were observed in all stages of development; the chromatin stained bright pink, and the pigment granules were clustered together. Within 6½ hours of the first dose of drug it was observed that, although young parasites were still normal in appearance, the chromatin of the larger forms was no longer in discrete particles and no mature parasites were seen. Twenty-two and a half hours after drug treatment had been started, all the parasites were of a large form, with indistinct, pale, and undivided chromatin; pigment granules were scattered throughout the parasites. At 30 hours the appearance of the parasites was much the same, and vacuolation of the cytoplasm was becoming apparent. At 46½ hours the parasites were found to be much vacuolated, with an irregular outline, and were degenerating. In the presence of drug, the degree of parasitaemia declined but little during the period of observation, while in the untreated birds the parasites multiplied normally and continued to show all stages of development with brightly staining chromatin.

In a similar experiment in which the chickens were treated with 4 mg. mepacrine it was found that the parasites reacted to the drug somewhat more quickly, and little evidence of parasitaemia was found 30 hours after the first dose of drug. In contrast to the effects produced by proguanil, mepacrine caused a shrinkage and vacuolation of the cytoplasm, followed by a contraction and fragmentation of the parasite to give a number of dark-staining masses of irregular shape.

Antimalarial Activity in vitro.—When maintained *in vitro* under the condition described above some parasites were able to survive for at least 40 hours. Observations on the increase in prepatent period showed that during 24 hours' incubation there was a loss in culture viability of about 60%, while for a 40-hour period of incubation the loss in viability was about 90%. Culture survival was, however, variable from experiment to experiment; with 24-hour cultures mortality varied from 0 to 90%, and with 40-hour cultures from 50 to 99.9%. Although the cultures showed a decrease in the number of viable parasites as incubation proceeded, it was nevertheless observed, from examination of stained films, that most of the parasites continued to develop *in vitro*. It appeared that parasites were able to grow and develop, in many cases to maturity, but on liberation from the mature schizonts the merozoites were usually unable to invade fresh red cells. Thus from this cause alone there would be a steady loss in viability of the "cultures."

Although the parasites in culture were clearly not under optimal conditions, the antimalarial effects of a number of substances could nevertheless be observed *in vitro*. In the presence of an active compound the mortality might be considerably increased and, moreover, the treated parasites often showed morphological differences from the control. Thus 0.5 mg./l. of mepacrine or chloroquine, or 20 mg./l. of pamaquin, were able to increase the prepatent period, compared with the control, by 1.2 days or more, showing that there had been at least a 90% mortality among the parasites which would have survived in the absence of drug. Furthermore, examination of stained preparations showed that the drugs had produced changes in the parasites similar to those

observed *in vivo*; the parasites were shrunken, dark-staining, and degenerate in appearance.

Proguanil and related compounds also showed antimalarial activity *in vitro*; morphological changes in the parasites were similar to those produced *in vivo*, and viability was usually reduced—though at higher drug concentrations than those necessary to produce morphological changes. These effects were noticeable in 24-hour cultures, and usually became more pronounced with further incubation. Proguanil, at concentrations of 5 mg./l. or more, reduced parasite viability by 90% compared with the control, and at concentrations down to 1 mg./l. usually caused the production of significant numbers of "inhibited" parasites, having pale, diffuse, and undivided chromatin. Differential parasite counts showed that the number of parasites with divided chromatin was very much smaller than that in the controls. The metabolite of proguanil, 10,580, was able to produce significant numbers of "inhibited" parasites in concentrations as low as 0.01 mg./l.; the lethal effects of this compound were rather variable.

5943 was found to be a little more active than proguanil, producing morphological changes in concentrations down to about 0.5 mg./l., and lethal effects down to about 5 mg./l. The metabolite 10,732 was the most active compound examined; lethal effects were apparent at a concentration of 0.01 mg./l., and inhibited parasites were produced by concentrations of 0.001 mg./l., and on occasions by even less than this. Pyrimethamine suppressed chromatin division, and led to the production of medium-sized forms with pale and diffuse chromatin in concentrations as low as 0.005 mg./l.; the lethal effect of this compound was rather variable, but concentrations of 0.5 mg./l. usually were sufficient to give a 90% increase in mortality.

With these low drug concentrations there was a suppression of chromatin division, although the parasites continue to increase in size, and a variable but significant number of large parasites with undivided, pale, and diffuse chromatin were produced. With high drug concentrations, of the order of 100 mg./l., the effect was of a different type, the parasites becoming shrunken, dark-staining, and degenerate, in a similar manner to those treated with mepacrine.

Attempts were made to prevent the *in vitro* antimalarial effects of proguanil and 10,732 by the addition to the cultures of pteroylglutamic acid and a number of other compounds. Proguanil was used at a concentration of 5 mg./l., and 10,732 at 0.013 mg./l. (4×10^{-8} M), concentrations which, on most occasions, produced typical effects on both morphology and viability. Pteroylglutamic acid (10^{-8}, 10^{-6} or 10^{-5} M), *Leuconostoc citrovorum* factor (10^{-8} or 10^{-6} M), adenosine (10 or 50 mg./l.), or a mixture of adenine (15 mg./l.), guanine (15 mg./l.), and thymine (10 mg./l.), were added to cultures in the presence of drug, but on no occasion was there any antagonism of antimalarial action due to the presence of these substances.

To give support to the contention that the drug effects observed *in vitro* were not artefacts, similar experiments were carried out using the resistant strain of the parasite. Proguanil and 5943 caused a shrinkage and degeneration of the parasite rather than the production of inhibited forms, while 10,580, 10,732, and pyrimethamine had little effect on morphology, except perhaps to cause a slight scattering of the pigment granules. Concentrations of drug up to 100 mg./l. were tested, and it was found that lethal effects were only produced at concentrations higher than those necessary with the normal strain. Thus proguanil was active at 10 mg./l., or more, and 5943 at 25 mg./l. 10,580 showed no lethal effect at 100 mg./l. in 24 hours, but was effective at 10 mg./l. after 40 hours' incubation. 10,732 was active at 10 mg./l. after 24 hours, or 1 mg./l. after 40 hours, while pyrimethamine showed corresponding activities at 30 mg. and 2.5 mg./l.

Discussion

It is now generally believed that pteroylglutamic acid is used by many living cells in the elaboration of a substance catalytically active in the synthesis of a number of purines, pyrimidines, and amino-acids. Using *Lactobacillus casei*, Hitchings, Falco, VanderWerff, Russell, and Elion (1952) studied the ability of a series of 2:4-diaminopyrimidines to antagonize PtG in the growth of this organism. They noted that one of the most active compounds of the series was pyrimethamine, which had an inhibition index of 2,300. The present work confirms the antagonistic action of this compound (inhibition index found 1,800), and shows that the triazines 10,580 and 10,732, which are similar in structure to pyrimethamine, are also active antagonists. When Falco *et al.* (1949) originally suggested a connection between antimalarial activity and the ability to antagonize PtG, they suggested that proguanil ought to show this antagonistic activity. Indeed, they demonstrated antagonism by PtG and also by purines. Although their curves show some reversal of drug toxicity against *L. casei* at certain proguanil concentrations, they do not demonstrate competitive

inhibition of the kind associated with pyrimethamine and the triazines. We have been unable to confirm the antagonistic action of proguanil, or of the dichloro analogue 5943; these compounds either produced a toxic action on the bacteria which could not be reversed by PtG, or had no toxic effect at all.

With the 2:4-diaminopyrimidines or diaminotriazines, one molecule of PtG is able to reverse the toxic action of several thousand molecules of drug. The present study shows that there is a more nearly equimolecular relationship between these compounds and thymine, and it seems likely that the drugs produce their antimalarial effect, not by antagonizing PtG but rather by interfering with reactions involving thymine or its derivatives. This being so, PtG would exert an antagonistic action, not by competing directly with the drug, but by acting catalytically in the synthesis of increased amounts of thymine.

Proguanil, the triazines, and diaminopyrimidines are all rather slow in producing a therapeutic response in cases of malaria, and can readily give rise to resistant strains of parasites. This seems to suggest that they exert their effects on the anabolic rather than on the catabolic systems of the parasites, and that growth of the parasite in the presence of drug is necessary for activity to be revealed. The activity of this group of drugs seems to involve an inhibition of nuclear growth and division. Black (1946) found that *P. falciparum,* maintained *in vitro* in the presence of serum from patients treated with proguanil, continued to develop to the early schizont stage, but that development then ceased, the chromatin failed to divide, and the parasites slowly degenerated. Thurston (1951) observed that the development of *P. berghei, in vivo,* in the presence of proguanil, was unaffected up to the schizont stage, but that normal nuclear division did not take place. Similarly McFadzean (1951) observed that schizogony of *P. cynomolgi* was inhibited in the presence of proguanil, abnormal forms containing elongate chromatin fragments being produced. The present study suggests that, with *P. gallinaceum,* proguanil, or its active metabolite, is able to inhibit the synthesis of nuclear material; both the parasites and their nuclei seem to increase in size in the presence of drug, but, as chromatin synthesis is inhibited, the nucleus fails to divide, and its staining power is decreased.

It is strange that Taylor, Josephson, Greenberg and Coatney (1952) were unable to find activity with proguanil in concentrations below 50 mg./l., or pyrimethamine at 100 mg./l. However, the effective drug concentrations in their experiments probably differed from ours, since their cultures contained whole blood rather than serum-Ringer-glucose-bicarbonate. In the present work proguanil showed detectable activity in concentrations as low as 1 mg./l., and pyrimethamine had something like 200 times this activity. These *in vitro* drug effects were of the same type, although not necessarily as pronounced as those observed *in vivo*; the difference in degree is probably accounted for by the parasites in culture being under conditions far less favourable to growth than those found in the host. The cultures showed about the same degree of survival found by Taylor *et al.* (1951) using the rocker-dilution technique, but our cultures contained rather more trophozoites and fewer schizonts than theirs. *In vitro,* the five compounds showed relative activities in the same order as that observed *in vivo,* but the differences in activity were greater; thus 10,732 showed 100 times the activity of proguanil *in vivo,* and 1,000 times the activity *in vitro.* This is doubtless due to the fact that the drugs act as such *in vitro,* whereas *in vivo* they are subject to metabolic changes by the host. It is significant that proguanil itself *in vitro* has quite a marked antimalarial action; the concentrations necessary to produce this effect, however, are about ten times greater than concentrations likely to be encountered *in vivo.*

Although drug toxicity in bacterial systems can be overcome by PtG or thymine, no such reversal was observed in the cultures of malaria parasites. The parasites are possibly unable to use PtG as such, but require a "higher form" of folic acid, and, rather than utilize free purines or pyrimidines in the synthesis of nucleic acids, prefer to utilize the corresponding nucleosides or nucleotides. Preliminary estimations suggest that both normal and parasitized cells contain the same amount of deoxyribonucleic acid phosphorus; this would seem to indicate that the parasites synthesize their chromatin at the expense of that of the host cell. It is possible that the utilization of the chromatin of the host cell does not involve a complete breakdown to purines, pyrimidines, phosphate, and deoxyribose, in which case free nitrogenous bases would not be expected to antagonize drug activity. Madinaveitia and Raventós (1949) made the interesting but inexplicable observation that the block in the auriculo-ventricular contraction of the guinea-pig heart produced by adenosine was antagonized by most antimalarials tested with the exception of proguanil; adenosine, however, showed no antagonistic effect towards any of the drugs tested in our malaria parasite cultures.

Summary

1. The metabolite of proguanil, 2:4-diamino-1-*p*-chlorophenyl-1:6-dihydro-6 : 6-dimethyl-1 : 3 : 5-triazine (10,580), the corresponding dichloro analogue (10,732) and pyrimethamine, are antagonists of pteroylglutamic acid and thymine in the growth of *Lactobacillus casei* ; no such antagonism is caused by proguanil itself or its dichloro analogue (5943).

2. Proguanil *in vivo* causes morphological changes in *Plasmodium gallinaceum* which suggest an inhibition of chromatin synthesis and nuclear division.

3. When proguanil (1 mg./l.), 5943 (0.5 mg./l.), 10,580 (0.01 mg./l.), 10,732 (0.001 mg./l.), or pyrimethamine (0.005 mg./l.) are incubated with erythrocytic cultures of *P. gallinaceum in vitro*, they cause morphological changes of the same type as those produced by these drugs *in vivo*. Drug concentrations about ten times as great are able *in vitro* to reduce culture viability by 90%.

4. A strain of *P. gallinaceum* made resistant to the maximum doses of proguanil tolerated by chickens showed resistance *in vitro* to proguanil, 5943, 10,580, 10,732, and pyrimethamine ; drug concentrations effective against the resistant strain *in vitro* did not produce morphological effects that would suggest an interference with chromatin synthesis.

5. The antimalarial effects of these drugs *in vitro* are not antagonized by pteroylglutamic acid, thymine, or a number of other substances.

I should like to thank Dr. D. G. Davey for much helpful advice and discussion during the course of this work, Drs. D. D. Woods and J. Lascelles for instruction in the methods of folic-acid assay, and Miss Margaret Mitchell for technical assistance.

Pyrimethamine was kindly supplied by Dr. J. W. Trevan, F.R.S., of the Wellcome Research Laboratories.

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KINETICS OF LACTIC DEHYDROGENASES OF *SCHISTOSOMA MANSONI* AND OF RABBIT MUSCLE*

BY

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(RECEIVED AUGUST 31, 1953)

It has been shown that in *Schistosoma mansoni* glycolysis proceeds at an exceedingly rapid rate (Bueding, 1950) and that this process is more critical for the survival of the parasite than is respiration (Bueding and Peters, 1951; Bueding, Peters, Koletsky, and Moore, 1953). Although glycolytic enzymes might catalyse the same reactions in the host as those in the parasite their nature might be different. Such differences would be of importance for the development of chemotherapeutic agents. They would afford opportunities to select specific inhibitors against enzymes of the parasite which would be without effect on those of the host. In an attempt to investigate such a possibility the kinetics of lactic dehydrogenase of *Schistosoma mansoni* were compared with those of lactic dehydrogenase of rabbit muscle.

METHODS

Adult worms were dissected from the mesenteric and portal veins of mice six to eight weeks after these animals had been infected with cercariae of *Schistosoma mansoni*. All subsequent operations were carried out at temperatures varying between 0 and 2° C. The worms were cut with fine scissors in distilled water (1 ml. of water was used per 100 pairs of worms). The process of cutting required two minutes. The suspension containing the worm fragments was stirred for five minutes in a beaker surrounded with cracked ice. This extract was then centrifuged for five minutes at 4,000 r.p.m., and 0.1 ml. of phosphate gel (dry weight per ml.: 1.4 mg.) was added per ml. of supernatant fluid. After stirring, this mixture was centrifuged for 10 minutes at 4,000 r.p.m. Most of the enzymatic activity was then absorbed by the use of 0.1 ml. of calcium phosphate gel (dry weight per ml.: 14 mg.) per ml. of solution. After centrifuging for 10 minutes at 4,000 r.p.m. the residue was washed with 0.01 M glycylglycine buffer (*p*H 7.5). Elution of the enzyme was carried out with a volume of solution containing glycylglycine buffer (0.5 M; *p*H 7.5) and potassium chloride (0.5 M) equivalent to one-half of the original extract. Sucrose was added to this eluate (final concentration: 32%) because this ensured the stability of the enzyme when stored in the frozen state. In a number of experiments a homogenate was used. This was prepared by homogenizing the worms in glycylglycine buffer (0.01 M; *p*H 7.5). After centrifugation for 10 minutes at 4,000 r.p.m. sucrose (final concentration: 32%) was added to the supernatant fluid which was used as the source of the enzyme.

The mammalian enzyme was prepared by the method of Kornberg and Pricer (1951). Reduced diphosphopyridine nucleotide ($DPNH_2$)† was prepared according to Gutcho and Stewart (1948), and obtained as a dry powder according to Ohlmeyer (1938). Enzymatic activity was measured by a spectrophotometric procedure described by Kornberg and Pricer (1951). This method is based on the change in the optical density at a wavelength of 340 mμ as a result of the oxidation or reduction of diphosphopyridine nucleotide (DPN)† during the following reaction:

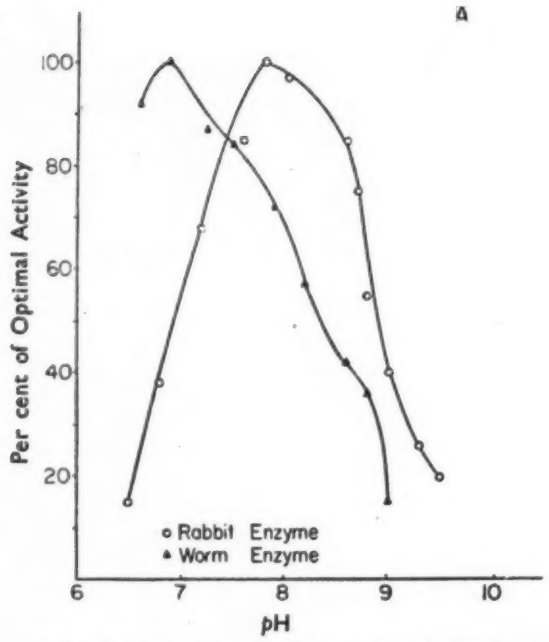
$$\text{Pyruvate} + DPNH_2 \rightleftarrows \text{lactate} + DPN$$

All measurements were made in a Beckman spectrophotometer in an air-conditioned room at 26° C. Under optimal conditions the activity of the enzyme was proportional to its concentration. The latter was adjusted so that under optimal conditions 0.013 to 0.016 μM of DPN was reduced or oxidized in one minute. The total volume of the reaction mixture was 1 ml. Reduction of DPN was recorded over a period of two minutes, and oxidation of DPN for one minute. When tested under optimal conditions, the specific lactic dehydrogenase activity of homogenates of schistosomes was two to three times lower than that of alkaline extracts of rabbit or rat muscle.

Lithium-*l*(+)lactate was prepared as follows: A commercial preparation of zinc-*l*(+)lactate (City Chemical Corporation) was dissolved in distilled water (16 ml. per g. of salt). The solution was run through a cation exchange resin (Dowex 50). The eluate,

* This investigation was supported by grants from the Office of Naval Research and from the Division of Research Grants and Fellowships, National Institutes of Health, U.S. Public Health Service.

† In this paper the following abbreviations are used: DPN—oxidized diphosphopyridine nucleotide; $DPNH_2$—reduced diphosphopyridine nucleotide.



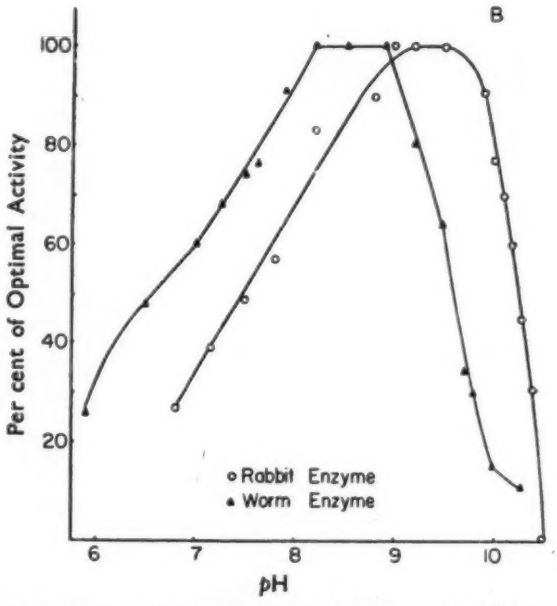
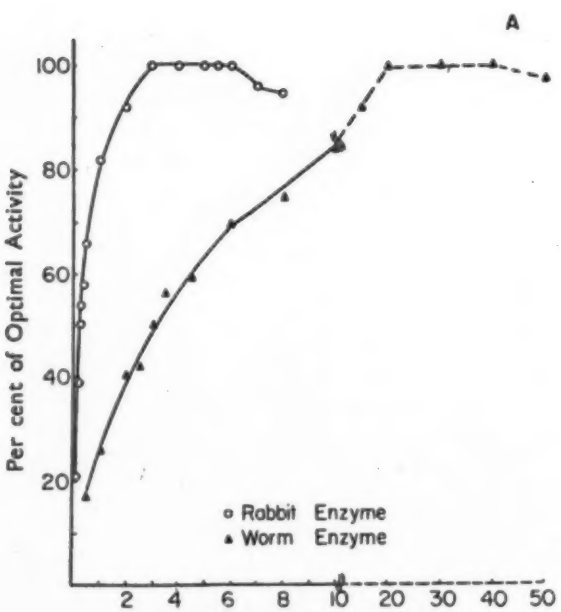


FIG. 1.—Effect of *p*H on the activities of the lactic dehydrogenases of schistosomes and of rabbit muscle. Abscissae: *p*H. Ordinates: Percentage of optimal activity.

FIG. 1A.—Reduction of pyruvate.

FIG. 1B.—Oxidation of lactate.



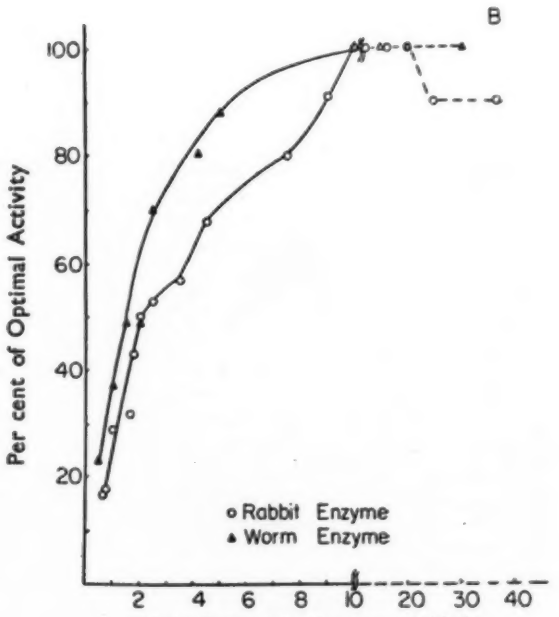


FIG. 2.—Effect of substrate concentration on the activities of the lactic dehydrogenases of schistosomes and of rabbit muscle. Ordinates: Percentage of optimal activity.

FIG. 2A.—Reduction of pyruvate. Abscissa: Molar concentration of pyruvate.

FIG. 2B.—Oxidation of lactate. Abscissa: Molar concentration of *l*(+)lactate.

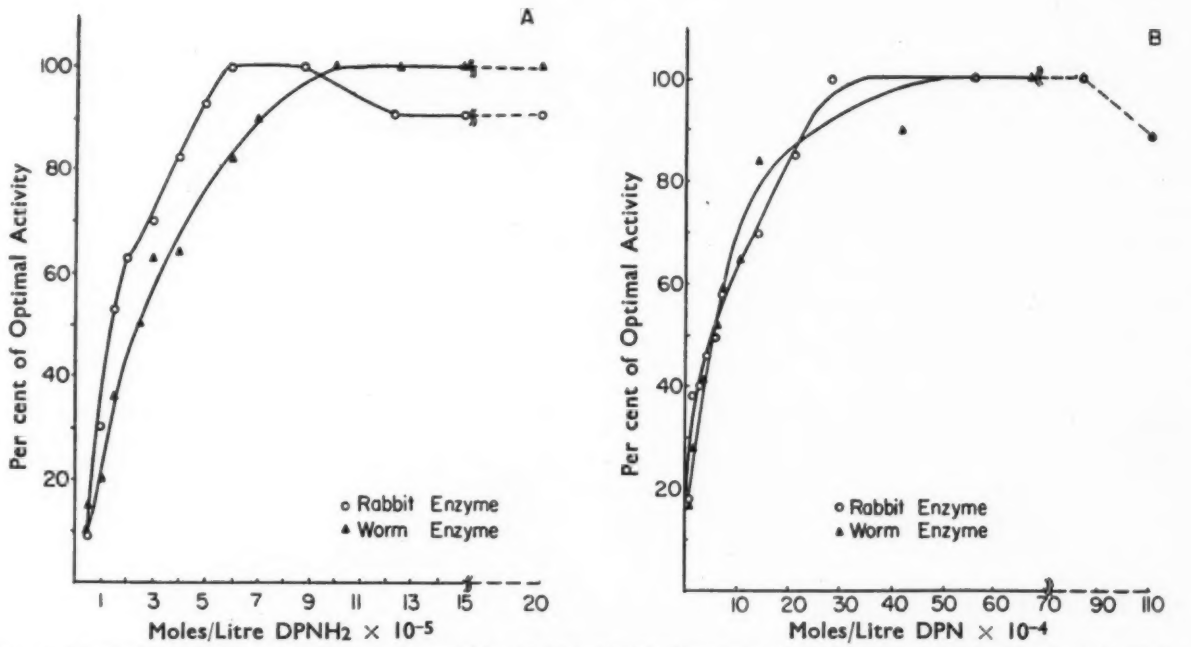


FIG. 3.—Effect of coenzyme concentration on the activities of the lactic dehydrogenases of *Schistosoma mansoni* and of rabbit muscle. Ordinates: Percentage of optimal activity.

FIG. 3A.—Reduction of pyruvate. Abscissa: Molar concentration of $DPNH_2$.

FIG. 3B.—Oxidation of lactate. Abscissa: Molar concentration of DPN.

which was free of zinc, was concentrated *in vacuo* and neutralized with lithium carbonate. Optical rotation of this solution revealed the absence of *d*(−) isomer. The latter was prepared according to Herzog and Slansky (1911). In the kinetic studies the *l*(+) lactate was used exclusively because no reduction of DPN occurred when *d*(−) lactate was used as a substrate.

RESULTS

Effect of p*H*.—The activities of both the schistosome and the mammalian enzymes were tested at different hydrogen ion concentrations in the presence of optimal concentrations of the substrates. Measurements of the rate of reduction of pyruvate to lactate revealed differences in the effect of *p*H on the activity of the two enzymes. The optimal activity of the worm enzyme was at *p*H 6.9 and that of the mammalian enzyme at *p*H 7.8 (Fig. 1A). The activity of the schistosome enzyme was tested also in the presence of phosphate buffer. Under these conditions optimal activity was seen even at lower *p*H levels. The optimal range for the oxidation of lactate to pyruvate was between *p*H 8.2 and 8.9 for the schistosome enzyme and between *p*H 9.0 and 9.5 for the mammalian enzyme (Fig. 1B). Therefore, the *p*H optima for the schistosome enzyme were significantly lower in either direction of the reaction.

Effect of Substrate Concentrations.—The effects of concentrations of $DPNH_2$ and pyruvate on one hand, and of DPN and lactate on the other, were determined for both enzymes. The results are shown graphically in Figs. 2 and 3. Table I summarizes the optimal concentrations and the Michaelis–Menten (1931) constants (K_M) of the substrates for both enzymes. It should be noticed that the dissociation constants and the optimal concentrations for lactate are identical for both enzymes. These two values were, however, six to twelve times higher for the worm enzymes with pyruvate, and slightly higher for the same enzyme with DPN and $DPNH_2$.

TABLE I

KINETIC DATA FOR THE LACTIC DEHYDROGENASES OF RABBIT MUSCLE AND OF *SCHISTOSOMA MANSONI*

All values are expressed as molar concentrations

Reaction	Substrate	Worm Enzyme		Rabbit Enzyme	
		Optimal Concentration	K_M	Optimal Concentration	K_M
Pyruvate→ lactate	$DPNH_2$	10×10^{-5}	$2{\cdot}5 \times 10^{-5}$	6×10^{-5}	$1{\cdot}5 \times 10^{-5}$
,, ,,	Pyruvate	20×10^{-3}	$3{\cdot}0 \times 10^{-3}$	3×10^{-3}	$0{\cdot}25 \times 10^{-3}$
Lactate→ pyruvate	DPN	56×10^{-4}	$5{\cdot}95 \times 10^{-4}$	28×10^{-4}	$5{\cdot}6 \times 10^{-4}$
,, ,,	*l*(+) lactate	10×10^{-2}	$2{\cdot}0 \times 10^{-2}$	10×10^{-2}	$2{\cdot}0 \times 10^{-2}$

Discussion

The differences in the kinetics of lactic dehydrogenase from rabbit muscle and from schistosomes suggest that the two enzymes are not identical, although they catalyse the same reaction. Similar kinetic differences between the enzymes of *Schistosoma mansoni* and those catalysing the same reaction in its mammalian host have been observed with hexokinase (Bueding and MacKinnon, 1953) and acetylcholine esterase (Bueding, 1952). It should be noted that the optimal substrate concentrations and the dissociation constants for the enzymes of schistosomes are usually higher than those for the host. This may be explained by a greater availability of substrate in the environment of the parasite, and may be related to a process of biochemical adaptation in an environment of high and relatively constant substrate concentration.

Summary

The kinetics of lactic dehydrogenase of *Schistosoma mansoni* are compared with those of lactic dehydrogenase of rabbit muscle. Differences in the *p*H optima and in some dissociation constants suggest that these two enzymes are not identical.

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THE PURIFICATION, POTENCY, AND AMINO ACID CONTENT OF MELANOPHORE-EXPANDING, PRESSOR, AND OXYTOCIC PREPARATIONS FROM BEEF PITUITARY GLAND

BY

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(RECEIVED JUNE 12, 1953)

Of the amino acid composition of the hormones of the posterior lobe of the pituitary gland least is known of that of the melanophore-expanding hormone. The need for more knowledge concerning this hormone has increased recently because of publications whose authors think it is related to ACTH. In this paper the amino acid composition of a melanophore preparation is presented, and some observations are made upon its relation to ACTH.

In recent years Du Vigneaud and his collaborators have made notable progress in isolating the pressor and oxytocic hormones of the pituitary gland and in determining the constituent amino acids of the products (Livermore and Du Vigneaud, 1949; Turner, Pierce, and Du Vigneaud, 1951; Popenoe, Pierce, Du Vigneaud, and Van Dyke, 1952; Pierce and Du Vigneaud, 1950a, b). Their method of isolation, countercurrent distribution, has yielded only small quantities of the hormones. For the analyses they used starch column chromatography.

In this paper the results of Du Vigneaud *et al.* are confirmed by entirely different methods. In addition, it is shown that these hormones may be obtained in larger amounts by a simpler method than countercurrent distribution.

Materials and Methods

Melanophore-expanding Hormone Preparation.—The preparation used was 150 times as potent as standard powder. A note by R. L. Stehle concerning its preparation will be found at the end of this paper.

The preparation was hydrolysed and the hydrolysate analysed by paper chromatography. From the intensities of the spots produced by the reaction with ninhydrin (observed by the eye directly) an attempt was made to get semi-quantitative information about the quantities of each acid. An effort to get more exact quantitative results by the use of a densitometer, as described by Block (1950), was abandoned after a test of its applicability using mixtures of pure amino acids.

A picrate like that from which this preparation was derived was also analysed. This picrate consisted of fine irregular crystals which decomposed at about 150–160°.

Pressor and Oxytocic Hormone Preparations.—The work was done partly on preparations put at the disposal of the writer by Dr. Stehle; these had been the subject of several communications by him and his collaborators. In addition, the author has isolated similar products from raw material following the technique of Stehle and Fraser (1935).

Paper Chromatography of the Unhydrolysed Preparations.—Pilot experiments were first carried out with 1-mg. amounts on Whatman No. 1 filter paper, and with 3-mg. amounts on Whatman No. 3 filter paper. One dimensional chromatography was by the ascending technique using a mixture of N-butanol glacial acetic acid, and water in the proportions 4:1:5. On developing the strip with ninhydrin various zones appeared, and the one containing the hormone was located by bioassay (Fig. 1 *a*). Two dimensional chromatography was carried out first in water-saturated phenol, and then in the mixture of N-butanol and acetic acid.

To obtain large quantities of the hormones paper chromatography on a larger scale was then applied, using 40 mg. of material on Whatman No. 3 filter paper, run in the mixture of N-butanol and acetic acid by the ascending technique. The material was dissolved in 0.1 ml. of 0.25% acetic acid and was placed on a line 20 cm. long in separate spots with a micropipette. It was run for 24 hours, after which the sheet was dried in air and cut in horizontal strips, using as a guide small vertical strips from both ends, which were developed with ninhydrin. The horizontal strips were then eluted with 0.25% acetic acid by running 10 ml. from a microburette through each strip during a period of not less than one hour. The solution was evaporated in a desiccator over calcium chloride and the residue dissolved in 0.5 ml. of water. After adding 5 ml. of methanol the material was precipitated with 45 ml. of ethylacetate.

Hydrolysis.—3–5 mg. was dissolved in 0.3–0.5 ml. 6 N-hydrochloric acid and heated in a sealed ampoule at 110–112° for 20 hours. The

content of the ampoule was then evaporated to dryness in a vacuum desiccator over calcium chloride and potassium hydroxide. The residue was dissolved in enough water to make a solution which contained about 0.15 mg. in 0.02 ml., this being the amount of solution placed upon the paper.

The picrate was treated in the same fashion ; the picric acid moved faster than the amino acids in all of the solvents, and did not affect their distribution.

Paper Chromatography of the Hydrolysed Preparations.—Chromatograms were made as follows :

1. Two dimensional, ascending, the first run with secondary butanol, 150 parts ; 3% ammonia, 60 parts. This was repeated. The 90° run was with secondary butanol, 150 parts ; 88% formic acid, 30 parts ; water, 20 parts (Hausmann, 1952). This procedure is suitable for alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, oxyproline, phenylalanine, proline, threonine, tyrosine, and valine.

2. One dimensional, ascending, with 90% formic acid, 15 parts ; water, 15 parts ; tertiary butanol, 70 parts. This is the technique of Block (1950) for cystine. The colour is developed with isatin (Acher, Fromegeot, and Jutisz, 1950) for the detection of oxyproline and proline.

3. One dimensional, descending, for five days, with water-saturated tertiary amyl alcohol, the water layer containing 1% diethylamine (Work, 1949). This is suitable for isoleucine, leucine, methionine, phenylalanine, and valine.

4. One dimensional, descending, for four days, with organic phase obtained by mixing normal butanol, acetic acid, and water in the ratio 4 : 1 : 5 (Partridge, 1948). For arginine, cystine, histidine, and lysine.

5. One dimensional, descending, for two days, with water-saturated phenol, the water layer containing 1% ammonia. Before starting, the cystine is oxidized to cysteic acid (Dent, 1947). For aspartic acid, cystine, glutamic acid, glycine, serine, and threonine.

The sheets were dried in air, sprayed with 0.1% ninhydrin in water-saturated normal butanol, and placed in the dark for 24 hours. To get an approximation to the amount of each amino acid from the colour intensities, the chromatograms were compared with those obtained with a 0.07 μM solution of amino acids in 0.6 N-hydrochloric acid.

Tryptophane was determined by the method of May and Rose (1922), as modified by Stehle (1945) to permit the use of small quantities of material.

Assays of Potency.—The pressor hormone was tested by measuring the blood pressure effect in dogs anaesthetized with chlorbutol.

Oxytocin was tested on the isolated uterus of the virgin guinea-pig suspended in Locke solution.

Results

Melanophore-expanding Hormone

The constituent amino acids of the melanophore hormone preparation are listed in Table I.

TABLE I

CONSTITUENT AMINO ACIDS OF THE MELANOPHORE-EXPANDING HORMONE

Present in largest amounts:	
Arginine	Proline
Glutamic acid	Serine
Glycine	Tryptophane
Histidine	Tyrosine
Lysine	Valine
Phenylalanine	
Present in smaller amounts:	
Alanine	Leucine
Aspartic acid	
Present in traces:	
Cystine	Methionine
Isoleucine	Threonine

Pressor and Oxytocic Hormones

The two dimensional chromatography of the crude preparations (Stehle and Fraser, 1935) gave the following picture (Fig. 1 *b*, *c*): R_f vasopressin in phenol, 0.85–0.90 ; in butanol-acetic acid, 0.15–0.18. R_f oxytocin in phenol, 0.85–0.90 ; in butanol-acetic acid, 0.35–0.40. Impurities—very probably lysine, arginine, and lower peptides—had an R_f value of 0.15–0.40 in phenol and 0.05–0.14 in butanol-acetic acid. If the ninhydrin-sprayed sheet was placed in an oven at 100° for a few minutes, the hormone spots gave a yellow colour that turned purple on prolonged heating. They gave a purple colour with ninhydrin on standing in the dark for 24 hours.

To remove the impurities, a one dimensional chromatography with 40 mg. of each of the impure preparations was carried out in butanol-acetic acid. The constituent amino acids of these preparations are listed in Tables II and III. The main constituents are identical with those reported by Du Vigneaud and his collaborators (Turner *et al.*, 1951 ; Popenoe *et al.*, 1952 ; Pierce *et al.*, 1950a, b). The spots of the amino acids present in traces were extremely faint in comparison with the others.

A picrate which consisted of fine irregular crystals was obtained from the pressor hormone.

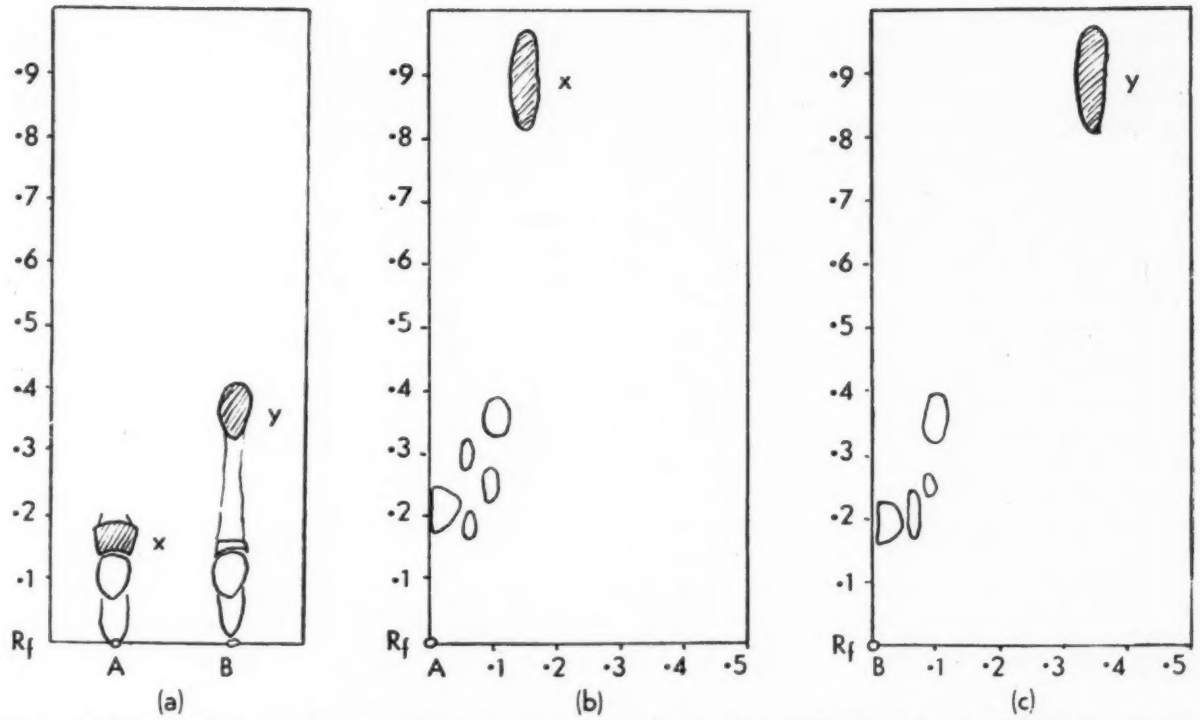

FIG. 1.—(*a*) One dimensional paper chromatography of the pressor and the oxytocic hormone (Stehle and Fraser, 1935) in N butanol-acetic acid-water, 4:1:5. A, point of application; x, pressor hormone after development. B, point of application; y, oxytocic hormone after development. (*b*) Two dimensional paper chromatography of the pressor hormone (Stehle and Fraser, 1935) in water-saturated phenol (vertical) and N butanol-acetic acid-water, 4:1:5 (horizontal). A, point of application; x, hormone. This material when purified by chromatography showed only the hormone spot. (*c*) Two dimensional paper chromatography of the oxytocic hormone (Stehle and Fraser, 1935) in water-saturated phenol (vertical) and N butanol-acetic acid-water, 4:1:5 (horizontal). B, point of application; y, hormone. This material when purified by chromatography showed only the hormone spot.

TABLE II

CONSTITUENT AMINO ACIDS OF THE PRESSOR HORMONE

Present in large amounts:

Arginine	Glycine
Aspartic acid	Phenylalanine
Cystine	Proline
Glutamic acid	Tyrosine

Present in traces:

Alanine	Leucine
Valine	

TABLE III

CONSTITUENT AMINO ACIDS OF THE OXYTOCIC HORMONE

Present in large amounts:

Aspartic acid	Isoleucine
Cystine	Leucine
Glutamic acid	Proline
Glycine	Tyrosine

Present in traces:

Alanine	Valine

It was prepared by adding a saturated aqueous solution of picric acid to a 1% solution of the pressor hormone; it precipitated instantly. The precipitate was separated by centrifugation and freed of excess picric acid by repeated washings with ethylacetate. It decomposed at 175–190°, and, when hydrolysed and chromatographed, gave the same chromatogram as the starting material.

The potency of the vasopressin was 200 units per mg. before chromatography and 330 units per mg. afterwards. This preparation had an oxytocic activity of about 15 units per mg. The potency of oxytocin was 250 units per mg. before chromatography, and remained unchanged by chromatography within the limits of error of the assay. 50 units had no pressor activity.

DISCUSSION

Melanophore-expanding Hormone

The conclusion of Sulman (1952) and of Johnsson and Hoegberg (1952), based upon the melanophore-expanding action of ACTH preparations, that the melanophore hormone is identical with ACTH has been denied by Geschwind, Reinhardt, and Li (1952); and by Reinhardt, Geschwind, Porath, and Li (1952). Recently, however, Brink, Boxer, Jelinek, Kuehl, Richter, and Folkers (1953), and Winter, Brink, and Folkers (1953), have stated that their corticotropin-B has a melanophore-expanding action; and since they claim that their material is pure the question appears to be still open.

It would seem that one way to try to settle the matter would be to test the corticotrophic action of the melanophore-expanding hormone, but this has not been done. The preparations which have had an action upon melanophores could have been contaminated preparations. Folkers *et al.* state that 0.1 μg. of their preparation expanded the melanophores of pieces of frog skin bathed in Ringer solution. Jores (1933), however, has found that 3.3 μg. of standard pituitary powder, which is equivalent to 0.022 μg. of the preparation studied in this work, is effective in December when the sensitivity is greatest. It is not stated when Folkers *et al.* made their tests. 0.01 μg. of the preparation studied in this work produced a strong effect, and 0.005 μg. produced an easily detectable effect when intact frogs, stored during the winter months, were injected in May. It would obviously not take much contamination of ACTH with such material to confer a melanophore-expanding action upon it.

Apart from testing the melanophore hormone for corticotrophic action, the solution of the problem might be advanced by giving attention to the tryptophane content of ACTH preparations. This amino acid seems to be a constituent of the melanophore hormone ; in preparations of different potencies the potencies run parallel to the tryptophane content (Stehle, 1945).

The amino acid constituents of ACTH as published (Geschwind, Porath, and Li, 1952 ; Li, 1952 ; Brink, Boxer, Jelinek, Kuehl, Richter, and Folkers, 1953), and of the melanophore hormone as given here, agree to a considerable extent except for tryptophane.

Pressor and Oxytocic Hormones

Biological assays of pituitary hormones have not a high degree of accuracy, and this may lead to claims of potency that are too high.

In their most recent publication, Pierce, Gordon, and Du Vigneaud (1952) conclude that, for their oxytocic hormone, 450–500 units per mg. is probably more nearly correct than the 800–865 units per mg. reported earlier (Livermore and Du Vigneaud, 1949 ; Pierce and Du Vigneaud, 1950b). On the other hand, they now state that their pressor preparation has 600–650 units per mg. (Popenoe, Pierce, Du Vigneaud, and Van Dyke, 1952) rather than the 400–450 units per mg. reported earlier (Turner, Pierce, and Du Vigneaud, 1951).

The potencies of the preparations described by Stehle and Fraser (1935) were given as 200 units per mg. for the pressor and 250 units for the oxytocic hormone. Dr. Stehle has informed the author that these potencies were given because his object was to assign values below which the potencies probably did not fall. Many individual assays gave higher values.

The pressor preparation mentioned above, which was obtained by chromatography from one of Stehle's preparations, and of which the chromatogram of the hydrolysate agreed with the composition given by Du Vigneaud, had only 330 units per mg. The corresponding oxytocic preparation had only 250 units per mg. If an underestimate of the potencies given for these preparations is as great as the overestimate of the oxytocic preparation first reported by Du Vigneaud, then his preparations and ours may be about equal in potency, and therefore in purity.

Summary

1. The component amino acids of a preparation of the melanophore-expanding hormone of the pituitary gland were determined. It appears to be distinguishable from ACTH by its content in tryptophane.

2. There is as yet no reason for believing that the melanophore hormone is identical with ACTH. Simple contamination of the latter with the former is an adequate explanation of the experimental results at present available.

3. A simple method is described for obtaining relatively large quantities of the pressor and oxytocic hormones of the pituitary gland in a highly purified state.

4. The amino acid composition of the products agrees with that given by Du Vigneaud *et al.*

5. The view is expressed that the potencies of these hormones may not be as high as is sometimes stated.

6. The pressor hormone may be precipitated as a crystalline picrate.

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ADDENDUM

by

R. L. STEHLE

This preparation was obtained in May, 1952, from a picrate similar to those described before (Stehle, 1944). After the removal of the picric acid 638 mg. of the material was dissolved in 25 ml. of water and 150 ml. absolute alcohol was added. Turbidity but no flocculation occurred. 50 ml. of ethylacetate was then added. The turbidity increased and the solution was put aside for the night. By morning a precipitate had formed. This was removed by centrifugation and dried *in vacuo* (184 mg.). Ethylacetate to make 500 ml. was added to the solution. A precipitate formed slowly and after several hours was removed by centrifuging and dried (313 mg.). Ethylacetate to make 900 ml. was then added to the solution. The next day the precipitate which had formed was centrifuged and dried (62 mg.). This last precipitate had about twice the tryptophane content and was about twice as potent upon frogs as was the 1944 preparation.

Lack of material prevented a repetition of the work, but from trials with such residues as were available it seemed clear that the outcome depends upon removing the second precipitate at the right time. If this is delayed too long the hormone comes down with it and one obtains no precipitate on final addition of ethylacetate. It was a surprise, and more or less a disappointment, to find that the potency of the 1944 preparation could be increased.

Brit. J. Pharmacol. (1953), **8**, 440.

RAT POISON FROM WHITE SQUILL

BY

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(RECEIVED JULY 11, 1953)

It is generally known that white squill contains little, if any, rat poison (for literature, see Gold, Modell, Catell, Benton, and Cotlove, 1947). However, as early as 1938, Dr. J. O. Pinkston, former chairman of this department, discovered that, whereas white squill bulbs collected from the coastal areas were inactive, those from the Lebanon mountains showed such activity that he used white squill powder to exterminate wild rats in and around the campus of the University. We have later obtained white squill from another part of the Lebanon mountains which occasionally showed as much activity as red squill. In general, however, the bulbs we have used yielded a dry squill powder that was lethal to male rats in an oral dose of 1.5–2 g./kg. body weight.

Attempts to isolate the active principle from white squill were begun in 1942. In 1944 a small quantity of crystalline material was obtained with an oral MLD in male rats of about 1.5 mg./kg. Owing to the war, the isolation of scilliroside from red squill by Stoll and Renz (1942) became known to us only in 1945. Although the two methods of preparation had nothing in common, the products appeared to be identical, judged by the results of elementary analysis, crystal form, solubility, and the Liebermann colour reaction.

In 1950 the Sandoz firm in Basle kindly supplied us with a sample of crystalline scilliroside, and a new batch of white squill was used to prepare material for comparison. It was soon found that the 1944 success had been dependent on the use of a brand of Merck (Darmstadt) charcoal which had stood in the laboratory for many years. The active substance was quantitatively adsorbed by the old charcoal and was eluted in a relatively pure form by treatment with 50% *iso*propyl alcohol. The available specimens of charcoal—Darco G 60, Mallinckrodt, and a new specimen from Merck—adsorbed the substance so tenaciously that it could not be eluted by 50% *iso*propyl alcohol, and the procedure had therefore to be modified from that step onwards. An amorphous colourless product, 30–35% as toxic to rats as Stoll's scilliroside, was obtained, and proved to be a mixture of scilliroside and a closely related product. This paper describes the properties of both compounds.

MATERIALS AND METHODS

Preparation of Rat Poison From White Squill.—The squill bulbs were collected during the months of June and July from a spot in Southern Lebanon *ca.* 1,800 ft. above sea level and about 15 miles from the Mediterranean coast. Activity was not lost if the bulbs were stored for a few months in a cool and shady place. Six kg. of intact scales was treated with 18 l. of a solution made by diluting 11.4 l. of 95% alcohol with 6.6 l. of water and kept standing at room temperature for a week. This solution contained maximum activity and a minimum of residue. The filtered solution was treated with lead subacetate until no more turbidity was produced on further addition. It was allowed to stand for not more than five hours, when the upper clear layer was siphoned off and the residue centrifuged. The clear fluid was immediately treated with enough sodium acid phosphate to remove the lead. The clear filtrate was then evaporated at a temperature of about 15° C. to approximately 1.5 l., and enough (*ca.* 40–60 g.) decolorizing charcoal added to adsorb all active material; it was kept in the refrigerator for 24 hours and shaken occasionally. The charcoal was filtered and washed with water and 95% alcohol. The alcohol-moist charcoal kept indefinitely in a glass-stoppered bottle.

Elution.—The old procedure need not be given in detail, since it used a sample of charcoal that is no longer available. It involved extraction of the charcoal five to six times with 50% *iso*propyl alcohol, evaporation of the extract to a small volume and repeated extraction of the aqueous residue with chloroform; evaporation of the chloroform extract to a small volume and adsorption on a column of alumina (Merck, standardized according to Brockmann); extrusion of the column and extraction with ice-cold 95% alcohol. The alcohol extract was evaporated to dryness and the residue crystallized from aqueous methanol in the form of irregular prisms.

(Found: C, 62.15; H, 7.84. Scilliroside, $C_{32}H_{44}O_{12}$, requires C, 61.92; H, 7.15%).

According to the new procedure the charcoal adsorbate was shaken for one hour with 500 ml. of a solution containing 75 volumes 95% alcohol, 20 volumes chloroform, and 5 volumes water. The extraction was repeated four or five times and the combined extracts evaporated at a low temperature to a volume of 80 ml. The brown and turbid residue was extracted several times with a total of 100 ml. benzene. From the benzene extract a crystalline aglucone was isolated which will be described elsewhere, but which possessed no lethal action on rats. The aqueous layer was evaporated to remove the benzene and then extracted four times with a total of 500 ml. of a chloroform solution containing 5% (v/v) butanol. This solution, according to Stoll and Renz (1942), extracts scilliroside but not scillaren. The combined extracts, which contained 85% of the activity, were evaporated to dryness under reduced pressure, dissolved in 25 ml. acetone, and adsorbed on a column of acid-washed alumina (Merck, Rahway, N.J.). The column was washed with 200 ml. acetone, and elution was accomplished with a total of 500 ml. 50% alcohol-acetone mixture. On evaporation the alcohol-acetone extract gave an amorphous, slightly coloured powder, which was then dissolved in ethyl acetate. This solution was evaporated until it became turbid and was then treated, while shaking, with an excess of petroleum ether. Ethyl acetate was removed by repeated decantations and replacement with petroleum ether. The product was filtered and dried. Repeated adsorption on alumina and elution with other fluid mixtures, such as 50% alcohol-ethyl acetate, or 10% methanol in chloroform, gave the same product and did not contribute to an increase in activity. The yield was about 1.5 g. from 6 kg. fresh scales.

Acetylation of the White Squill Mixture.—The usual method, utilizing acetic anhydride and pyridine at room temperature, was employed (Stoll and Renz, 1942). The product was purified on the alumina column.

Oxidation of the Acetylated Product.—The method of Stoll, Renz, and Helfenstein (1943) was used.

Separation of the Mixture by Paper Chromatography.—Water was used as the stable phase and a mixture of cyclohexane and cyclohexanol as the mobile phase.

Paper Electrophoresis.—This was kindly performed by Dozent Dr. Kurt Wallenfels, Boehringer & Sons, Mannheim, Germany. The paper was sprayed with perchloric and sulphuric acids and heated prior to inspection under the ultra-violet lamp.

Acid Hydrolysis of the White Squill Mixture.—The method of Mannich and Siewert (1942) was used, utilizing hydrochloric acid and acetone at room temperature for two days. The details of the purification of the two crystalline aglucones obtained will be published elsewhere.

Results

Analyses.—1. *White squill mixture* dried in vacuum at 80° C. Found:—Batch I: C, 62.0; H, 7.47. Batch II: C, 62.64; H, 7.57. Scilliroside ($C_{32}H_{44}O_{12}$) requires C, 61.92; H, 7.15%.

2. *Acetylated product* dried in high vacuum at 80° C., m.p. 131–133° C. Found: C, 60.54; H, 6.61. Tetraacetyl-scilliroside ($C_{40}H_{52}O_{16}$) requires C, 60.90; H, 6.65%.

3. *Oxidized acetylated product,* m.p. 233–234° C. No depression with compound prepared from crystalline scilliroside. Found: C, 61.33; H, 6.47. Dehydrotetraacetyl-scilliroside ($C_{40}H_{50}O_{16}$) requires C, 61.04; H, 6.41%.

4. *Hydrolysis products of white squill mixture.* Aglucone I—needles, from methanol, m.p. 223–225° C. Found: (i) C, 73.66; H, 7.76; (ii) C, 73.41; H, 7.45%. Aglucone II—leaflets, from ethanol, m.p. 232–234° C. Found: C, 76.33; H, 8.01%.

Paper Chromatography.—This revealed one zone for scilliroside and two contiguous zones for the white squill mixture, one of which moved at the same rate as scilliroside.

Paper Electrophoresis.—This also revealed one band for scilliroside and two for the white squill mixture.

Ultra-violet Absorption Spectra.—The spectrum of the white squill mixture and that of scilliroside are identical. Both show a maximum at 297 mμ and a molecular extinction coefficient of 3.71 at that wavelength. (For complete curve see Stoll and Renz (1942)).

Liebermann Colour Reaction.—Stoll's scilliroside: violet→blue→blue-green. White squill mixture: violet→blue→blue-green.

Toxicity to Male Rats.—It can be seen from Table I that the white squill mixture is 30–35% as active as scilliroside.

TABLE I

COMPARATIVE TOXICITIES OF SCILLIROSIDE AND WHITE SQUILL RAT POISON BY INTRAVENOUS INJECTION TO MALE RATS

Scilliroside			White Squill Product		
Dose mg./kg.	No. of Rats	No. Died with Convulsions	Dose mg./kg	No. of Rats	No. Died with Convulsions
0·8	6	0	1·8	6	0
0·9	12	6	2·5	6	2
1·0	18	14	2·7	12	5
			3·0	12	10

Cardiotoxic Activity (Table II).—Dog heart-lung preparations were used and the glycosides were infused at the optimal rate according to Farah (1946). Before the infusion was started the hearts were made hypodynamic by pentobarbitone; the initial systemic output was decreased to one half or less. The therapeutic effect was assessed by a decrease in right atrial pressure and a simultaneous increase in systemic output. It will be noticed that scilliroside is less active than ouabain but more active than digoxin, digitoxin, and oleandrin, as judged by the data obtained by Farah and Maresh (1948). It will also be seen from Tables I and II that although the white squill glycoside mixture contains only 30–35% scilliroside it is about 55% as toxic to the heart, indicating that the rat-inactive product is cardiotoxic but less so than scilliroside. The ratio of the therapeutic dose to the lethal dose is slightly higher for the squill glycosides than that obtained by Farah and Maresh for other heart poisons. This may be due to the fact that we have used a big dose of pentobarbitone to induce failure, and in most experiments have started the infusion of the glycosides while the atrial pressure was still rising. Where the therapeutic dose is left out it means that the turning point was not sharp or that no definite therapeutic activity was observed.

TABLE II

THERAPEUTIC AND LETHAL DOSES OF SQUILL GLYCOSIDES IN DOG HEART-LUNG PREPARATIONS

In all except the first four experiments of the first series, pentobarbitone was added to reduce the initial systemic output to one half or less, before infusion of the glycosides was started

Exp. No.	Glycoside	Rate of Administration. Micromoles/min	Therapeutic Dose in % of Lethal Dose	Lethal Dose. Micromoles/kg. Heart	Duration of Infusion of Glycoside in Min.
1A	Scilliroside	0·002	—	3·41	80
2A	,,	0·002	—	4·10	88
3A	,,	0·002	—	3·54	79
4A	,,	0·002	30·4	3·51	102
5A	,,	0·002	—	3·89	96
6A	,,	0·002	23·6	2·94	102
7A	,,	0·002	32·6	3·95	92
8A	,,	0·002	22·7	2·79	88
9A	,,	0·002	13·8	2·38	94
10A	,,	0·002	13·4	2·62	112
			Av. 22·7	3·31	93·3
1B	,,	0·001	20·1	3·42	174
2B	,,	0·001	20·2	2·1	148
3B	,,	0·001	15·1	3·3	165
4B	,,	0·001	20·8	3·96	231
			Av. 19	3·19	179
	White squill mixture product M1b				
1C	,, ,,	0·004	23·1	5·86	108
2C	,, ,,	0·004	—	4·77	85
3C	,, ,,	0·004	27·8	4·40	90
4C	,, ,,	0·004	22·2	6·92	108
5C	,, ,,	0·004	—	8·45	97
			Av. 24·4	6·08	98
1D	,, ,,	0·002	21·65	6·30	162
2D	,, ,,	0·002	29·20	5·24	137
3D	,, ,,	0·002	26·00	5·88	154
4D	,, ,,	0·002	—	5·84	158
			Av. 25·6	5·81	153
	White squill mixture product M1c				
1E	,, ,,	0·002	24	6·94	150
2E	,, ,,	0·002	28	5·0	160
3E	,, ,,	0·002	27·2	7·73	184
4E	,, ,,	0·002	18·2	5·67	165
5E	,, ,,	0·002	30·6	6·23	163
6E	,, ,,	0·002	24·8	3·93	121
			Av. 25·4	5·92	157

DISCUSSION

The amorphous product from white squill appears to be a mixture of two substances. This is indicated by the results of paper chromatography as well as paper electrophoresis experiments, which revealed only two zones. One of these products is clearly scilliroside, for oxidation of the acetylated mixture with chromic acid or lead tetraacetate according to Stoll, Renz, and Helfenstein (1943) gave, in a yield of 30%, a crystalline dehydrotetraacetyl-scilliroside which was indistinguishable from the product obtained from pure scilliroside. Since the amorphous product is only 30–35% as toxic to rats as scilliroside, it is difficult to avoid the conclusion that the rodenticidal activity of white squill is due exclusively to its scilliroside content, as Gold *et al.* (1947) had suspected. However, tested on the dog heart-lung preparation, the white squill mixture is about 55% as toxic as scilliroside, and we prefer to explain this by suggesting that the rat-inactive principle is toxic to the heart but less so than scilliroside. There are, however, alternative explanations, but we consider these to be less plausible. Assuming always that the methods of bioassay are sufficiently accurate, one could suspect, for instance, that both the rodenticidal and the cardiotoxic activities of the white squill mixture are due to its scilliroside content which might be, say, 55%. One would then have to assume that the preparative losses reduce the yield of dehydrotetraacetyl-scilliroside to 30% and that the rat-inactive principle interfered with the rat assay.

The nature of the rat-inactive principle is not known with certainty yet. But it must be closely related to scilliroside: the two products behave similarly on the alumina column, and have almost identical elementary compositions and molecular extinction coefficients. Acid hydrolysis of the amorphous product according to Mannich and Siewert (1942) yields two crystalline aglucones

which must be derived from the rat-inactive glycoside, since this method of hydrolysis yields no crystalline products from pure scilliroside. The chemical structure of these aglucones is being further investigated and will be published elsewhere. In the meantime further attempts are being made, by the use of paper chromatography, to isolate the rat-inactive principle on a preparative scale.

It is not known why the squill that grows in the coastal areas is almost devoid of lethal action on rats while that which grows in the mountains is active. The two are botanically indistinguishable. Three factors may be responsible for this difference—the soil, the altitude, and the nearness to the sea. Attempts are being made to solve this interesting problem by transplanting squill bulbs from the coast to the mountain area and vice-versa, both with and without the surrounding native soil.

Summary

1. Wild white squill that grows in some parts of the Lebanon mountains contains sufficient rat poison to be of use in exterminating rats. The white squill that grows in the coastal areas is almost inactive.

2. The active principle has been shown to be identical with scilliroside, the substance obtained from red squill grown in North Africa.

3. Scilliroside was tested, on the dog heart-lung preparation, for its cardiotoxic activity and was found to be less active than ouabain but more active than digoxin, digitoxin, and oleandrin.

4. White squill also contains another glycoside, hitherto undescribed, which bears a close chemical relationship to scilliroside, but which is not toxic to rats and is less cardiotoxic than scilliroside.

This study has been supported by a grant from the American Heart Association. We are greatly indebted to Dr. Alfred E. Farah for his advice and help in the early part of this work, and to Merck & Co., Rahway, N.J., U.S.A., for a generous supply of acid-washed alumina.

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Brit. J. Pharmacol. (1953), **8**, 444.

THE ACTION OF 2 : 4-DIAMINO-6 : 7-DIBENZYLPTERIDINE AGAINST ANTIBIOTIC-RESISTANT STAPHYLOCOCCI

BY

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(RECEIVED MAY 29, 1953)

2 : 4-Diamino-6 : 7-dibenzylpteridine was found (Collier and Waterhouse, 1952) to inhibit the growth of *Streptococcus faecalis* and *Staphylococcus aureus* when present in low concentration in the culture medium and to protect mice against infections of *Staph. aureus*; this action appeared to be potentiated by sulphathiazole. The therapeutic activity of the pteridine was not as high as that of penicillin against normal staphylococcal infections of mice; but we thought it desirable to explore its activity alone, and with sulphathiazole, against staphylococci resistant to penicillin and other antibacterial drugs.

MATERIALS AND METHODS

Strains.—Drug-resistant strains of *Staph. aureus* were obtained from three sources.

1. By serial transfer in the laboratory of strain CN491 in liquid media containing increasing concentrations of penicillin, sulphathiazole, dibenzylpteridine, or a mixture of equal weights of the two last-named.

2. Penicillin-resistant strains isolated by Dr. M. Barber at St. Thomas's Hospital, London, numbered D1, MID1, MID3, and W499.

3. Multiple-resistant strains Nos. 22 and 159 relatively insensitive to penicillin, streptomycin, aureomycin, chloramphenicol, terramycin, and sulphonamides, isolated at the Bristol Royal Infirmary (Clarke, Dalgleish, and Gillespie, 1952).

Methods in vitro.—The medium in all experiments had the following composition.

NaCl	5.0 g.
Na_2HPO_4	3.5 g.
KH_2PO_4	0.4 g.
"Vitamin free" casein hydrolysate (A. & H.) ...	5.0 g.
dl-Tryptophane	0.2 g.
Dextrose	2.0 g.
Aneurine	40 μg.
Nicotinic acid	400 μg.
Distilled water	to 1 litre
to *p*H 7.2–7.4	

In the induction of drug-resistance, inocula were adjusted to give approximately 30,000 cells per ml. experimental medium. In sensitivity tests inocula of this size or of 1,000,000 cells per ml. of test medium were used. End-points were read visually and the minimal inhibitory concentrations expressed as the geometric means of several independent experiments. For other details, reference may be made to Collier and Waterhouse, 1952.

Methods in vivo.—Methods used in mice were as described in the above paper, except that the infective inocula were prepared from 5-hr. cultures of staphylococci. Rabbits were infected intravenously with 0.5 ml. of a 5-hr. culture of staphylococci washed and made up to initial volume. 1 and 24 hours after inoculation, we administered dibenzylpteridine or sulphathiazole, or their mixture in equal proportions, by mouth, or procaine penicillin in oil with aluminium stearate by intramuscular injection.

RESULTS

The Development of Resistance in vitro

In the course of 10–12 transfers, in liquid medium containing increasing concentrations of drug, *Staph. aureus* CN491 became more than a hundred times more resistant to penicillin, sulphathiazole, or dibenzylpteridine, but only about six times more resistant to a mixture of equal parts of sulphathiazole and pteridine (see Fig. 1). In these experiments an inoculum giving approximately 30,000 cells per ml. of test medium was used.

Cross-resistance in vitro

Artificially Produced Drug-resistant Strains of Staph. aureus *CN491.*—Using washed inocula of about 30,000 cells per ml. culture medium, we found that strains made resistant to any one of the three drugs remained sensitive to the other two. Each of these resistant strains was sensitive to the pteridine-sulphathiazole mixture.

Naturally Occurring Penicillin-resistant Strains.—Since penicillin-resistance may be more clearly seen when large inocula of staphylococci are used

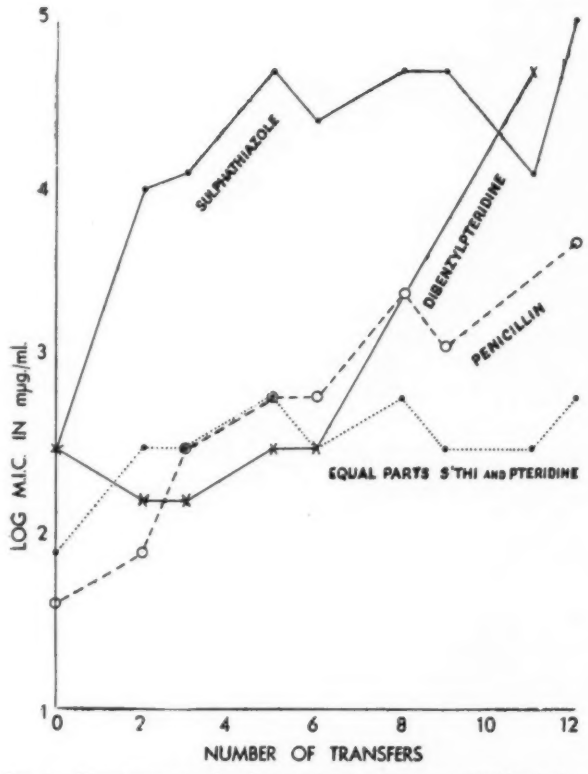

FIG. 1.—Development of resistance by *Staph. aureus* CN491 during exposure to sulphathiazole, dibenzylpteridine, and penicillin in serial subcultures. Ordinate: logarithm of the minimal inhibitory concentration (M.I.C.) in mμg./ml. Abscissa: number of transfers.

(see Barber, 1947) we employed inocula of about 1,000,000 as well as of about 30,000 washed cells per ml. medium when testing the cross-resistance of Barber's strains. The results of these experiments, illustrated in Table I, confirm the importance of inoculum size in the demonstration of penicillin resistance. They also show that, when a large inoculum was used, strain W499 exhibited a marked degree of resistance to dibenzylpteridine, but a mixture of sulphathiazole and dibenzylpteridine was effective against large inocula of this as well as of the other strains.

TABLE I

RESPONSES OF PENICILLIN-RESISTANT STAPHYLOCOCCI TO DIBENZYLPTERIDINE, SULPHATHIAZOLE, AND A MIXTURE OF THESE DRUGS, *IN VITRO*

The cultures were inoculated to give (*a*) about 1,000 000 cells/ml. and (*b*) about 30,000 cells/ml. respectively

Strain No.	Minimal Inhibitory Concentration of Drug in μg./ml. at 24 hr.						
	Penicillin		Dibenzyl-pteridine		Sulpha-thiazole		Pteri-dine+ S'thi. 50:50
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)
D 1, ..	25·12	0·36	2·33	0·25	>100	1·26	0·45
MID1	8·91	0·5	0·79	0·05	>25	0·45	0·31
MID3	>10	0·63	2·5	0·16	>100	1·25	0·45
W499 ..	>100	0·78	>100	0·16	>100	0·35	1·1

Naturally Occurring Multiple-resistant Strains.—Even using an inoculum of the smaller size (*ca.* 30,000 cells per ml. medium) we found that strains 22 and 159 exhibited considerable resistance to penicillin, chloramphenicol, and sulphathiazole. Accordingly, using the same sized inoculum, we explored their sensitivity to dibenzylpteridine and to a pteridine-sulphathiazole mixture, with results as expressed in Table II. It will be seen that both strains are sensitive to dibenzylpteridine, but a significantly greater sensitiveness to the pteridine-sulphathiazole mixture is not shown.

TABLE II

RESPONSES OF MULTIPLE-RESISTANT STAPHYLOCOCCI TO VARIOUS DRUGS *IN VITRO*

Cultures inoculated to give about 30,000 cells/ml.

Strain No.	Drug	Minimal Inhibitory Concentration in μg./ml.	
		24 hr.	48 hr.
22	Chloramphenicol	50	50
	Sulphathiazole	50	71
	Penicillin	12·5	>100
	Dibenzylpteridine	0·552	0·78
	Pteridine+S'thi. 50:50 ..	0·78	0·78
159	Chloramphenicol	50	71
	Sulphathiazole	100	100–>100
	Penicillin	12·5	100
	Dibenzylpteridine	0·552	0 78
	Pteridine+S'thi. 50:50 ..	0·552	1·1

Therapeutic Experiments

Naturally Occurring Penicillin-resistant Strains.—The strains from Dr. Barber were not pathogenic to mice, but one of them, W499, was rapidly fatal to rabbits when given intravenously. In spite of the resistance of W499 to dibenzylpteridine, shown *in vitro,* we carried out some preliminary experiments on the protection of rabbits. In these experiments oral doses of 1.0 g. of sulphathiazole or of dibenzylpteridine failed to protect the animals. A mixture of equal parts of the two drugs, on the other hand, prolonged the lives of rabbits at doses of 0.2 and 1.0 g. per kg. However, in spite of the penicillin-resistance exhibited by this strain *in vitro,* an intramuscular dose of 100,000 units of procaine penicillin in oil per kg. protected the animals longer than did the pteridine-sulphonamide mixture.

Naturally Occurring Multiple-resistant Strains.—Strains 22 and 159 proved capable of killing

mice if inoculated intraperitoneally in large numbers with 5% mucin. In preliminary experiments we found that, given by mouth, sulphathiazole had little protective activity and that a sulphathiazole-pteridine mixture was no better than the pteridine alone.

The pteridine itself showed activity when given orally in large doses, but it was less active against either strain than chloramphenicol, in spite of the moderate degree of chloramphenicol resistance. This is illustrated in Fig. 2, which gives the results of an experiment with strain 159.

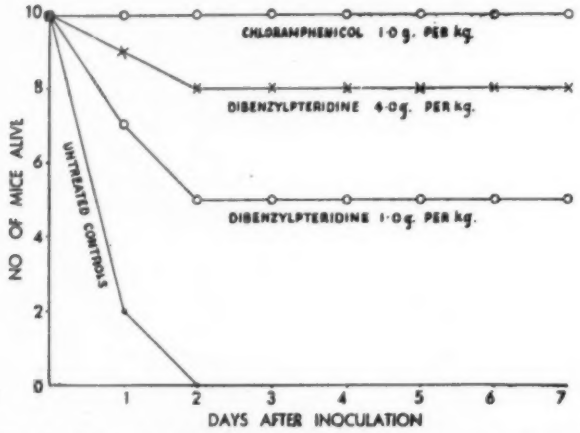

FIG. 2.—Protection of mice, infected with a multiple-resistant strain of *Staph. aureus* 159, by chloramphenicol and dibenzylpteridine administered orally 1 hr. after intraperitoneal inoculation. Ordinate: number of mice alive. Abscissa: days after inoculation.

The results of therapeutic tests in mice and in rabbits showed that dibenzylpteridine was less effective *in vivo* than might have been expected from its *in vitro* activity, while the converse was true of penicillin and chloramphenicol. One of the questions which therefore arose was how far body fluids antagonized the antibacterial activity of the pteridine.

TABLE III

ANTAGONISM OF DIBENZYLPTERIDINE, SULPHATHIAZOLE, AND THEIR MIXTURE BY VARIOUS SERA

Strain W499 inoculated to give about 30,000 cells/ml.

Drug	Serum Included at 5% in Synthetic Medium	Minimal Inhibitory Concentration in μg./ml. at 16 hr.
Dibenzylpteridine ..	None	0·046
	Rabbit	2·5–>10
	Mouse	0·88
	Man	1·25
Sulphathiazole ..	None	0·625
	Rabbit	>10
	Man	>10
Equal parts .. Dibenzylpteridine + Sulphathiazole ..	None	0·039
	Rabbit	0·625
	Man	0·625

Effect of Serum on Drug Activity

When 5% serum from man, mouse, or rabbit was added to the synthetic medium, strain W499 showed a marked decrease in sensitiveness to dibenzylpteridine, to sulphathiazole, and to a mixture of the two drugs. As will be seen from Table III, the antagonistic effect was least with a mixture of pteridine and sulphathiazole.

DISCUSSION

The experimental work *in vitro*, illustrated in Fig. 1, provides yet another example of resistance developing more slowly to a mixture of drugs than to one alone, as has been described for other drugs by Carpenter, Bahn, Ackerman, and Stokinger (1945) and more recently by, for example, Purcell, Wright, and Finland (1953).

Collier and Waterhouse (1952) showed that dibenzylpteridine exerted some therapeutic effect in mice against an antibiotic-sensitive strain of *Staph. aureus*. By the oral route, its activity was slight, but it was considerably more effective when given intraperitoneally. In the present therapeutic tests with antibiotic-resistant strains, the pteridine showed about the same activity by mouth as before. The intraperitoneal route of administration was not used, as it was thought that there might be some direct action of the drug on the staphylococci before their absorption into the blood stream. The potentiation of dibenzylpteridine by sulphathiazole, previously reported by Collier and Waterhouse for a normal strain of staphylococcus, was also found in those of the present strains of staphylococci not already resistant to sulphathiazole.

The relative ineffectiveness of the dibenzylpteridine *in vivo*, as compared with its performance *in vitro* in a synthetic medium, is at least partly explained by the antagonistic action of serum. This finding contrasts with the failure of oxalated horse blood to antagonize markedly the action of pteridines against *Str. faecalis* (Collier and Waterhouse, 1952).

SUMMARY

1. Strains of *Staph. aureus* made separately resistant in the laboratory to sulphathiazole, penicillin, and dibenzylpteridine respectively, showed no cross-resistance.

2. Exposure of serial subcultures to a mixture of dibenzylpteridine and sulphathiazole produced resistance less readily than did exposure to either drug separately, or to penicillin alone.

3. Resistance to dibenzylpteridine was found in one of four naturally occurring penicillin-resistant strains.

4. Two naturally occurring strains resistant to various antibiotics and to sulphonamides were sensitive to dibenzylpteridine.

5. A mixture of dibenzylpteridine and sulphathiazole given by mouth protected rabbits against infection with a moderately penicillin-resistant strain of *Staph. aureus*. Penicillin was also effective when given intramuscularly in high doses.

6. Dibenzylpteridine protected mice against two multiple-resistant strains of *Staph aureus*, but was less effective than chloramphenicol, when both drugs were administered by mouth.

7. Some antagonism of dibenzylpteridine by serum was demonstrated *in vitro*.

We wish to thank Miss M. Boothroyd and Mrs. E. Harris for technical assistance, and the directors of Messrs. Allen & Hanburys Ltd. for permission to publish this paper. The dibenzylpteridine was kindly prepared by Mr. J. H. Dunsmuir.

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CHEMOTHERAPEUTIC STUDIES ON THE VIRUSES OF SHEEP ABORTION AND MOUSE PNEUMONITIS (NIGG) WITH SPECIAL REFERENCE TO AN EMBRYONATED EGG METHOD

BY

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WITH AN APPENDIX BY LÖIS DICKINSON

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(RECEIVED JANUARY 8, 1952)

As part of a programme of research into the chemotherapy of virus infections, the need arose for a simple preliminary test which would select from numerous compounds, often available in only small amounts, those which might be of value in the chemotherapy of virus disease. The use of experimental virus infections in embryonated eggs is now widely accepted for such purposes and several techniques have been described. The aim of this work was to develop one such technique and to establish its value by using compounds known to affect virus proliferation—a condition which restricted investigation to viruses of the psittacosis-lymphogranuloma group (Findlay, 1951).

In investigating this group of viruses in embryonated eggs most workers have grown the virus in the yolk sac, into which they have also introduced the compound under investigation, and have measured antiviral action in terms of the extended life of the embryo. The practical disadvantages of this method are that frequent inspection of the embryo is necessary to determine the time of death, and that death must be demonstrated as being due specifically to virus multiplication. This latter usually involves the staining and examination of yolk sac smears—a somewhat tedious process.

These disadvantages could be overcome if a virus typical of the psittacosis-lymphogranuloma group, which would produce well-defined lesions susceptible of visual assessment, could be grown on the chorioallantoic membrane, the compound being given by some alternative route. For reasons of convenience the choice of virus was directed to those not pathogenic to man. Earlier attempts to establish mouse pneumonitis (Nigg) virus, by serial passage on the chorioallantoic membrane of virus from mouse lung, were discouraging. Learning of this difficulty, Mr. W. M. Brownlee, a veterinary colleague, aware of the discovery of a virus associated with ewe abortion (Stamp, McEwen, Watt, and Nisbet, 1950), drew attention to its possible suitability and obtained pathological specimens from a local case of the disease. The virus isolated from this material produced well-defined lesions on the chorioallantoic membrane and so was used to establish the test described. The use of this organism has been previously described (Dickinson and Inkley, 1951).

Results obtained from the embryonated egg test have been compared with results obtained by Dr. Dickinson using Nigg virus in mice. This has made possible some quantitative assessment of the embryonated egg test. It is assumed that the use of mice in chemotherapeutic studies is generally accepted as providing information relevant to disease in human beings. Details of the mouse-tests are given in the Appendix.

MATERIALS AND METHODS

Abortion Virus

Primary Isolation of the Virus of Sheep Abortion.—Smears of the foetal membranes, stained by Machiavello's stain, contained numerous elementary bodies, particularly in the region of the cotyledons. Portions of the cotyledons were ground in lemco broth to give an approximately 10% tissue suspension and centrifuged at 1,000 r.p.m. for 10 min. Streptomycin was added to the supernatant fluid to make a final concentration of 1,000 u./ml.; penicillin was avoided, since at this stage it was felt that the organism might be penicillin sensitive.

The inoculum thus prepared was placed on to the chorioallantoic membranes of 10-day embryos (prepared by the dropped-membrane technique); after a further 7-day incubation at 37° the membranes

were covered with discrete small lesions (Fig. 1). An inoculum for a second passage was made by grinding one of these infected membranes in 5 ml. lemco broth, centrifuging the suspension so prepared at 1,000 r.p.m. for 10 min., and then filtering the supernatant fluid through a gradocol membrane of pore size 1 μ; lesions were produced by dilutions of 1/10 and 1/100 of this filtrate.

Properties of Abortion Virus.—These are described by Stamp *et al.* (1950); in addition the following properties commend its use as a test organism.

(1) *Culture.*—After 20 passages on chorioallantoic membranes there was no apparent alteration in the lesions induced by the virus. Extremely high yields of virus were produced by yolk sac inoculation.

(2) *Freeze Drying.*—Virus suspensions, prepared by grinding infected chorioallantoic membranes or infected yolk sacs in broth, were successfully freeze-dried; the yolk sac suspensions proved particularly effective in maintaining the viability of the virus.

(3) *Viability.*—Infected whole chorioallantoic membranes suffered little loss of viable virus when kept at $-20°$ or $-70°$ for several months. Suspensions of infected yolk sac material in broth remained viable for months at $-70°$ and were valuable as stock inocula for test purposes.

(4) *Response of Host.*—Uniform lesion patterns on the chorioallantoic membrane were consistently formed and were readily assessed visually.

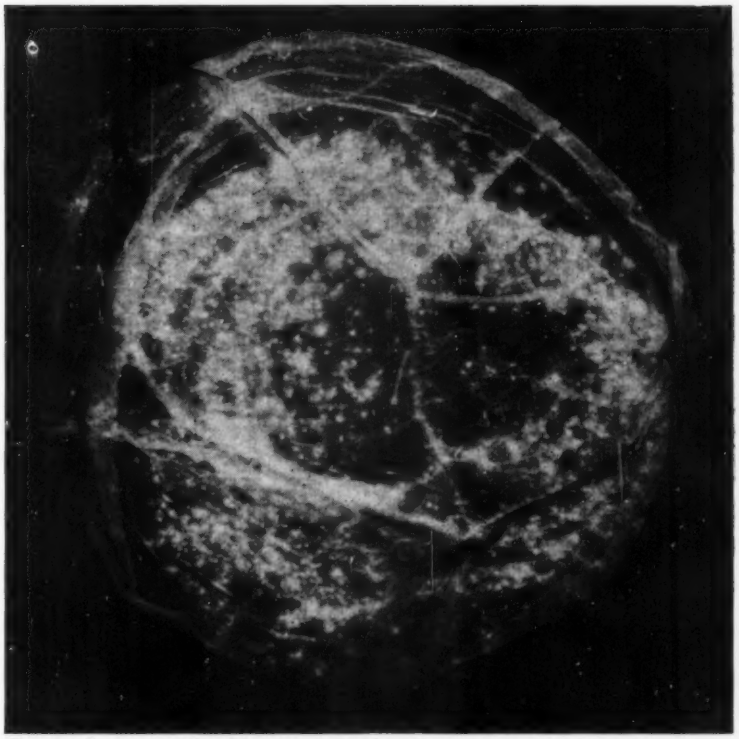

FIG. 1.—Lesions produced by sheep abortion virus on the chorioallantoic membrane (7 days' incubation). Magnification ×2.

Nigg Virus

Well-defined lesions on the chorioallantoic membrane were eventually obtained by the following procedure. Yolk sacs of 10-day embryos were infected with Nigg virus, obtained by gradocol filtration (pore size 1 μ) of a suspension of infected mouse lung. On death, each yolk sac was ground with 10 ml. broth and centrifuged at 2,000 r.p.m. for 10 min. The virus-rich supernatant material was collected, the fatty layer being avoided, and this provided a suitable inoculum when diluted 1/10 in lemco broth. These yolk sac inocula, unlike those of abortion virus, were relatively labile and did not produce the desired lesion pattern when inoculated directly on to the chorioallantoic membrane after storing for 2–3 weeks at $-70°$; they kept sufficiently well, however, to provide seed for yolk sac infection when fresh material was required (approximately one year at $-70°$). Their viability was maintained on freeze drying.

Route for Drug-inoculation

Since the virus was to be grown on the chorioallantoic membrane, only the route for drug-inoculation had to be decided. For practical reasons it had to be either the yolk sac or the allantoic sac. To compare the two routes the antibiotics aureomycin, chloramphenicol, and oxytetracycline ("terramycin") were used.

11-day embryos were given 0.2 ml. of aqueous two-fold dilutions of the drugs by one or other route. Abortion virus was placed on to the dropped chorioallantoic membrane 2–3 hr. later. Groups of six embryos were used at each dose level. Incubation was continued for a further 7 days. Distinct end points were obtained.

TABLE I

RELATIVE EFFICACIES OF DRUGS BY DIFFERENT ROUTES OF ADMINISTRATION

Drug	Inhibitory Dose* in mg./Egg	
	When Given by the Yolk Sac	When Given by the Allantoic Sac
Oxytetracycline ..	0·062	0·25
Aureomycin ..	0·25	1·0
Chloramphenicol ..	1·0	1·0

* Dose which prevents the formation of lesions on the chorioallantoic membrane. All compounds were toxic at 4·0 mg./egg.

As is shown in Table I, aureomycin and oxytetracycline are both more effective when given via the yolk sac. Some four times the amount of drug must be given into the allantoic sac to achieve the result obtained by yolk sac inoculation. With chloramphenicol, however, this does not hold, and both routes

are equally effective. Although no explanation is offered, the results are consistent with the known physiological functions of the allantoic sac and yolk sac.

The superior sensitivity of yolk sac inoculation made this the method of choice. Subsequently, when it was found that 200 u. each of penicillin and streptomycin given by the yolk sac did not affect abortion virus or Nigg virus, the various compounds under test were dissolved or suspended in an aqueous penicillin–streptomycin solution (1,000 u. of each/ml.). This practice is of great value, since many substances are difficult to sterilize and the yolk sac is particularly susceptible to infection by bacteria.

Method Finally Adopted

Embryonated egg techniques are described by Beveridge and Burnet (1946). For convenience, the modifications found useful are described in detail.

Embryonated eggs of 11 days' incubation are candled, and on each a position, just clear of the main allantoic vein, is marked on the shell, so as to be on the greatest diameter of the egg; diametrically opposite this a second mark is made to indicate the position for yolk sac inoculation; the air space is also marked. It is convenient to use only eggs in which the air space is at the "broad end" (normal). The shell is drilled for the yolk sac inoculation, sufficiently widely to permit the passage of a No. 12 hypodermic needle; the exposed shell membrane is clarified by one drop of hot wax (70°) which is then flattened to a very thin layer with a hot metal spatula. This latter procedure results in a sterile inoculation area and provides a good base for the subsequent wax seal. While awaiting introduction of the compound, this waxed area is kept downwards in order to orientate the yolk sac suitably. 0.2 ml. of a solution or suspension of the compound in water, containing 1,000 u./ml. each of penicillin and streptomycin, is then introduced into the yolk sac. For this the waxed area is brought uppermost and the needle thrust sharply 1 in. into the egg, which is transilluminated from the end opposite the air space. The yolk sac is displayed quite distinctly and inoculation can be seen to be successful. Occasionally, on withdrawing the needle, a small amount of allantoic fluid escapes but readily runs off the waxed area. The hole is then sealed with hot wax.

The egg, now containing the test compound, is drilled at the air space and a small triangle of shell is removed from the position marked near the allantoic vein. The chorioallantoic membrane is then "dropped" by wetting the exposed triangle of shell membrane with one drop of sterile broth and gently parting the fibres with a ball-ended glass rod. The chorioallantoic membrane usually falls away without further treatment. (Non-specific lesions resulting from this operation are extremely rare. The most common fault of the novice is the piercing of the chorioallantoic membrane, with the subsequent formation of a bubble of air in the allantoic cavity; in experienced hands this event is rare and about 100 eggs can be prepared in 20–30 min.) The virus inoculum is then deposited on to the "dropped" membrane, usually some 4–5 hr. after introducing the compound under test. The triangle of shell membrane is sealed with 3 drops of hot wax, pressed on with a warm spatula to give a perfect seal. In early work the inoculum was 0.2 ml. of a broth suspension prepared by grinding a fresh infected membrane in broth and centrifuging for 10 min. at 2,000 r.p.m., the supernatant fluid being made up to 20 ml. with broth. More recently, yolk sac suspensions, distributed in 1-ml. amounts and kept at −70°, have proved more convenient and are diluted up to 20–25 ml. in broth when required. Even distribution of the virus is ensured by gently raising in turn each side of the tray containing the sealed eggs.

The eggs are then incubated for a further 7 days at 37° and inspected daily to judge deaths resulting from the toxic effect of the various compounds; this latter is not really necessary unless detailed information on toxicity is required. Twofold dilutions of the compound, to include the dose toxic to the embryo, are tested; groups of six embryos at each level are sufficient to provide information.

At the end of the test period, the eggs are opened under 2% formalin by cutting the shell completely in half, the half containing the area of "dropped" membrane being retained. The membranes are stripped away from the shell and examined in petri-dishes. Lesions are readily obvious whilst stripping, and membranes bearing them can be discarded and the compound involved designated inactive. More careful scrutiny is applied to those with less distinct lesions. The embryos are not killed by the growth of the virus on the chorioallantoic membrane, and deaths can be related to the toxicity of the compound under investigation. It is not customary to examine the membranes from embryos which have died.

Results

Several compounds, previously described as active against viruses of the psittacosis-lymphogranuloma group under various conditions and in clinical use, have been examined by the embryonated egg test against both sheep abortion virus and Nigg virus. The results of the tests are summarized in Table II. Most of the figures were obtained from series in which twofold dilutions of drug were employed. It can be taken, where a figure is given as showing inhibition, that a dose of half that magnitude was inactive. A more exact measurement was not sought. Even so it is apparent that the order of effectiveness against both viruses is, on a weight basis, oxytetracycline > aureomycin > chloramphenicol. Both viruses appear to be equally sensitive to oxytetracycline and aureomycin, but Nigg virus seems rather more resistant to chloramphenicol than does the virus of sheep abortion.

TABLE II
EFFECT OF DRUGS ON VIRUS INFECTIONS IN EMBRYONATED EGGS AND IN MICE

Drug	Inhibitory Dose* (mg./Egg)		*Activity in Mice Against Nigg Virus (Drug given Subcutaneously from Day of Infection)†
	Abortion Virus	Nigg Virus	
Oxytetracycline ..	0·06	0·06	+ + + (0·02 mg./g./day)
Aureomycin	0·25	0·25	+ + + (0·02 mg./g./day)
Chloramphenicol ..	1·00	2·00	+ + (0·02 mg./g./day)
Penicillin ..	Inactive 6·66 ≡10,000 u.	1·33 ≡2,000 u.	+ (0·13 mg./g./day) ≡4,000 u./mouse/day
Sulphathiazole	Inactive 14·7	14·7	+ (0·2 mg./g./day)
p-Aminobenzoic acid	Inactive 27·4	Inactive 27·4	0 (2·0 mg./g./day) orally

* Dose which prevents the formation of lesions on the chorioallantoic membrane.
† +, prolongation of life only; + +, no deaths, but lungs involved; + + +, no deaths, lungs not involved.

Nigg virus is sensitive to penicillin and sulphathiazole. Abortion virus is not affected by penicillin and sulphathiazole at comparable concentrations: with penicillin, indeed, even five times the amount which inhibits Nigg virus is without effect. Neither virus is sensitive to *p*-aminobenzoic acid.

It is further evident from Table II that, for Nigg virus, the results given by the *in ovo* test closely follow those obtained from the mouse test. Details of the mouse test will be found in the Appendix and Table III.

Tests were also done in which the virus was established some time before the drug was introduced. For routine tests, however, this is undesirable, on account of the extra hazards inherent in the manipulation of infected eggs. Results from tests in which the infection had been established for as long as 24 hr. were similar to those in which the infection followed drug inoculation; but the mortality rate of embryos was very high, no doubt due to interference with respiration from the slowly absorbed liquid inoculum distributed around the chorioallantoic membrane.

DISCUSSION

In establishing the test described here for the examination of compounds for antiviral action, evidence has been obtained that *in ovo* tests are of value in chemotherapeutic studies of viral infections. The quantitative nature of the test is well demonstrated for Nigg virus, which may be considered a natural infection of mice. The disease, experimentally induced in mice by this virus, responds favourably to several of the compounds tested; the response of the *in ovo* infection to these same compounds is always of a like order. Several workers have studied other viruses on the chorioallantoic membrane, but none, so far as I am aware, were in a position to justify the method, since therapeutic agents active against the viruses they used were not available.

There is reason to suppose, in view of the physiological nature of the yolk sac, that a compound given into this organ is carried by the blood circulation to the embryo and then to the chorioallantoic membrane where it may be excreted into the allantoic sac. Thus the embryo itself experiences the toxic effects of the compound before it reaches the virus in the chorioallantoic membrane, so that the method affords a good measure of the therapeutic index of the drug—relative to the chick embryo. This may or may not be similar to the index obtained with a mammalian species. In contrast, when both compound and virus are introduced into the yolk sac the conditions favour the compound and, to this extent, such a test is more sensitive in determining the drug effect. The unfortunate result in practice is that compounds may appear to be active by *in ovo* tests and yet prove ineffective by animal tests, so that *in ovo* tests come to be held in disregard. It is against such a tendency that this paper is largely directed, since, in the present state of viral chemotherapy, the embryonated egg serves as the most powerful available tool in a field of investigation which must inevitably be somewhat empirical.

The use of abortion virus as a test organism in the search for chemotherapeutic agents against larger viruses suffers the disadvantages inherent in the use of any organism regarded as typical of a group. It would be better if attention could be directed to the particular disease organism for which therapy is sought. As a convenient experimental tool, however, the abortion virus possesses advantages, despite the fact that its sensitivity to chemotherapeutic agents is somewhat different from that of the Nigg virus.

SUMMARY

1. An embryonated egg test for the detection of antiviral agents is described. Its advantages and limitations are discussed.

2. Several compounds, known to affect the multiplication of large viruses, have been examined by this method against the viruses of sheep abortion and mouse pneumonitis (Nigg).

3. A comparison of the egg test and a mouse test (described in the Appendix) has been effected for Nigg virus, and a correlation which is markedly quantitative has been demonstrated.

4. The isolation of the strain of abortion virus used is described, and its advantages as a test organism set forth.

5. The value of embryonated egg tests in the present stage of viral chemotherapy is re-emphasized.

The author is indebted to Dr. Löis Dickinson for the primary isolation of the strain of abortion virus used in this work; for her keen interest in the development of this test; and for permission to quote the results of her mouse experiments, given in detail in the Appendix.

He is also indebted to Mr. W. M. Brownlee for his help in obtaining the pathological specimens; Dr. A. D. McEwen for helpful information on the abortion virus; Mr. G. Whiting for the photography; and the late Sir Jack Drummond, F.R.S., and Mr. C. E. Coulthard, who, by their keen interest, encouraged this work.

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APPENDIX

TESTS AGAINST MOUSE PNEUMONITIS (NIGG) VIRUS IN MICE

By Löis Dickinson

In vivo tests against mouse pneumonitis (Nigg) virus were carried out in order to compare the results of the *in ovo* test, described in the paper, with results obtained when the compounds were tested against the infection in mice produced by this organism.

Methods

0.05 ml. of a suspension of Nigg virus in lemco broth was given intranasally to groups of 20-g. albino mice under light anaesthesia. The stock virus consisted of infected lungs ground with 5 ml. broth per lung. This material was mixed with an equal volume of 10% glucose and freeze-dried in 1-ml. amounts. For tests, each ampoule was made up to 10 ml. and used undiluted, preliminary titrations indicating that this amount would kill most mice and infect all. All mice receiving 1/10th this dose showed infected lungs after 12 days, but only about 25% died (Table III). In every experiment the control group was the last group to be infected.

Treatment was commenced 1 hr. before infection, except where otherwise stated, and drugs were given twice daily, either subcutaneously or by stomach tube, for 12 days. Survivors were killed at 12 days and the lungs examined for evidence of infection. Lungs showing total consolidation were assessed at 4 (5 if death had occurred) and values of 3, 2, and 1 given for lesser consolidation. When the drugs had a pronounced effect on survival time, several mice were left on test for 10 days after the cessation of treatment to see whether the disease "flared up."

Table III

DRUG ACTION AGAINST NIGG VIRUS IN MICE

Drug	Dose/Day in mg./g.	Survivors at 12 Days	Survivors' Average Lung Assessment	Flare-up Test	
				Deaths	Infected Lungs of Survivors
Test 1	*(a) Normal Infecting Dose of Virus*				
Penicillin	1,000 u./mouse (S.C.)	8/10	2·3		
Controls		1/10	4·6		
	(b) High Infecting Dose of Virus				
Penicillin	1,000 u./mouse (S.C.)	2/18	—		
Controls		1/18	—		
Test 2					
Penicillin	4,000 u./mouse (S.C.)	15/19	3·0	4/7	3/3
Sulphathiazole	0·4 (S.C.)	18/20	1·6	4/8	3/4
,,	0·2 ,,	16/20	0·5	—	—
p-Aminobenzoic acid	2·0 (oral)	3/10	3·5	—	—
Control		6/20	3·4	—	—
Test 3					
Chloramphenicol	0·2 (oral)	11/11	—	0/11	3/11
,,	0·02 ,,	10/10	1·1	—	—
,,	0·02 (S.C.)	7/8	1·0	—	—
Aureomycin	0·1 (oral)	10/10	—	0/10	0/10
,,	0·02 ,,	10/10	0	—	—
,,	0·02 (S.C.)	9/9	0	—	—
Oxytetracycline	0·05 (oral)	10/10	—	0/10	0/10
,,	0·02 ,,	10/10	0	—	—
,,	0·02 (S.C.)	1 /10	0	—	—
,,	0·02 (neutra')	10/11	0	—	—
Contro's		4/12	3·0	—	—
Controls (1/10th infecting dose of virus)		8/12	2·1	—	—
Test 4	*Drugs for Four Oral Doses Starting One Day after Infection*				
Chloramphenicol	0·8 (total dose)	8/10	2·1	—	—
Aureomycin	0·4 ,, ,,	9/10	1·7	—	—
Oxytetracycline	0·2 ,, ,,	10/10	1·5	—	—
Controls		4/12	3·0	—	—

Results

All results are given in Table III and indicate the efficacy of oxytetracycline ("terramycin"), aueromycin, and chloramphenicol in that order. All these drugs suppressed the development of lung lesions, but a higher dose of chloramphenicol was required. Both oxytetracycline and aureomycin were effective at 0.02 mg./g./day orally or subcutaneously, and prevented the development of the

disease for 10 days after treatment had ceased, at 0.05 and 0.1 mg./g./day respectively. Previous work had shown that 0.02 mg./g. aureomycin was not effective against a high infecting dose of the virus and 0.01 mg./g. did not protect mice significantly in the normal test.

When treatment was limited to four doses starting one day after infection, all three drugs still exerted some protective action. A total dose of 0.8 mg./g. choramphenicol was less effective than 0.4 mg./g. aureomycin or 0.2 mg./g. oxytetracycline; there were no deaths in the oxytetracycline group, but the lungs showed evidence of infection.

The results with penicillin indicate the necessity for giving the maximum dose if an effect is to be noted; the effect was not observed when 400 u./mouse was given, and even 1,000 u./mouse had no effect if the infecting dose of virus was high. This drug only delayed death and did not markedly suppress lung lesions.

Sulphathiazole suppressed lung lesions to some extent, but was not nearly so effective as the antibiotics. *p*-Aminobenzoic acid was inactive.

Brit. J. Pharmacol. (1953), **8**, 454.

THYROID AND HYPERGLYCAEMIA PRODUCED BY ADRENALINE AND NORADRENALINE

BY

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(RECEIVED JULY 31, 1953)

The hyperglycaemia produced in rabbits by subcutaneous injection of adrenaline has often been stated to be augmented by thyroid feeding. There have been repeated suggestions that the destruction of adrenaline in the body, in some organs at least, depends on the action of the enzyme amine oxidase, and Burn (1952a) has recently emphasized findings which are consistent with this idea. The possibility thus arose that the augmentation of adrenaline hyperglycaemia by thyroid feeding might be due to a diminution in the amount of amine oxidase in the liver. Spinks and Burn (1952) found that a fall in amine oxidase took place after two weeks of thyroid feeding; and, further, that the operation of thyroidectomy, both in rabbits and in rats, was followed by a rise in the amount of amine oxidase in the liver.

The fall in liver amine oxidase was not large, and it was impossible to say with certainty that it accounted for the increase in adrenaline hyperglycaemia. Now noradrenaline is less effective than adrenaline in causing hyperglycaemia, as was first shown by Schümann (1949). Noradrenaline is also less effective than adrenaline in constricting the vessels of the rabbit ear, in dilating the pupil of the cat's eye, and in contracting the nictitating membrane: Burn and Robinson (1951) explained these observations by their finding that noradrenaline is more readily oxidized by amine oxidase than is adrenaline. Such an explanation would attribute the smaller hyperglycaemic action of noradrenaline to more rapid destruction by amine oxidase in the liver. Thus, thyroid feeding might be expected to augment noradrenaline hyperglycaemia to a greater extent than it augments adrenaline hyperglycaemia

Experiments have therefore been carried out to determine:

(1) the dose-response curve for the hyperglycaemia produced by adrenaline in normal rabbits;

(2) the amounts of adrenaline and noradrenaline equiactive in producing hyperglycaemia in normal rabbits;

(3) the effect of thyroid feeding on the hyperglycaemia produced by amounts of (*a*) adrenaline, (*b*) noradrenaline, which were roughly equivalent in normal rabbits;

(4) the effect of thyroid feeding on the amine oxidase in rabbit livers;

(5) the effect of small doses of ephedrine—which is known to inhibit amine oxidase—on the hyperglycaemia produced by adrenaline.

METHODS

Rabbits of various breeds and both sexes were taken at random from stock. Their weight varied from 1.5–2.5 kg. They were fed on a mixed diet of bran and whole cereal with addition of some cabbage. For thyroid feeding, dried thyroid was given by stomach tube for a period of 14 days with 1–2 days' interval, so that the daily intake amounted to 200 mg. irrespective of body weight.

Determination of Blood Sugar.—For an experiment six rabbits were kept without food for 18 hr. Blood was removed from the ear veins, mixed with small amounts of potassium oxalate, and the blood sugar estimated by a combination of Somogyi's (1930) method of precipitating the proteins with Hagedorn-Jensen's (1923) method of sugar estimation. Each determination was done in duplicate and the mean calculated. For the estimation of the initial value 2×0.5 ml. blood was removed; after the subcutaneous injection of adrenaline or noradrenaline 2×0.1 ml. blood was taken at intervals of 1, 2, 3, 4, and 5 hr. and the samples pooled. In half of the rabbits adrenaline was tested on the 13th day of thyroid-feeding, and noradrenaline on the 15th day. In the other half the order was reversed. The means were taken to be valid for the 14th day. A corresponding cross-over method was applied in the control experiments before thyroid feeding began. With one exception not more than two experiments were done within a week. All values refer to L-adrenaline and to L-noradrenaline.

Determination of Amine Oxidase.—Rabbits were stunned by a blow on the head and bled to death. The left liver lobe was removed immediately and tested at once or kept at −10° C. for not more than two days. For the manometric determination the method described by Spinks and Burn (1952) was adopted which consisted of the incubation of 50 mg. homogenized liver suspended in phosphate buffer (*p*H 7.4) with M/50 tyramine in the presence of M/100 semicarbazide and N-KOH (in the centre well). The oxygen uptake was measured for 30 min. and plotted in 5-min. intervals. The uptake between the 5th and 20th min. after tipping was measured and calculated as μl. O_2/100 mg. liver/15 min.

RESULTS

Dose-response Curve for Adrenaline.—The effect of a given amount of adrenaline or noradrenaline was measured by subtracting the initial blood sugar from the mean value during the five hours after injection, and expressing this difference as a percentage of the initial blood sugar. In order to find a suitable way to measure an increase in sensitivity, the relation between dose and response was determined. The details of the experiments are given in Table I. Fig. 1 relates the mean effect in six normal rabbits to each of five doses of adrenaline, plotted on a logarithmic scale. Statistical calculation showed that the regression line did not depart significantly from linearity. Over this dose range doubling the dose always increased the response by 41.3%.

TABLE I

HYPERGLYCAEMIC ACTION OF ADRENALINE IN NORMAL RABBITS EXPRESSED AS PERCENTAGE BLOOD SUGAR INCREASE

Rabbit	Dose of Adrenaline (s.c.) in μg./kg.				
	30	60	120	240	480
1	7·3	11·6	49·1	59·8	161·7
2	29·8	51·2	48·9	90·6	158·6
3	17·3	76·5	72·7	165·1	227·0
4	7·3	78·7	77·1	154·2	178·2
5	3·5	19·8	93·4	104·8	162·0
6	4·9	41·8	80·0	128·6	210·8
Mean	11·7	46·6	70·2	117.2	183·1

Adrenaline and Noradrenaline Equivalence.—Since Ellis (1951) found that the dose-response curve for noradrenaline was parallel to that for adrenaline, a dose-response curve for noradrenaline was not determined on so many rabbits as was that for adrenaline. Experiments in four rabbits showed that the mean percentage blood sugar rise was 24.1 for 125 μg./kg., 35.8 for 250 μg./kg., and 57.9 for 500 μg./kg.

The relation between the hyperglycaemic effect of adrenaline and noradrenaline was determined from observations in 14 rabbits which were tested both with 60 μg./kg. adrenaline and with 250 μg./kg. noradrenaline. These doses were chosen because they produced effects of a similar order. The results are given in Table II (rabbits Nos. 7–20). The mean effect of the injection of

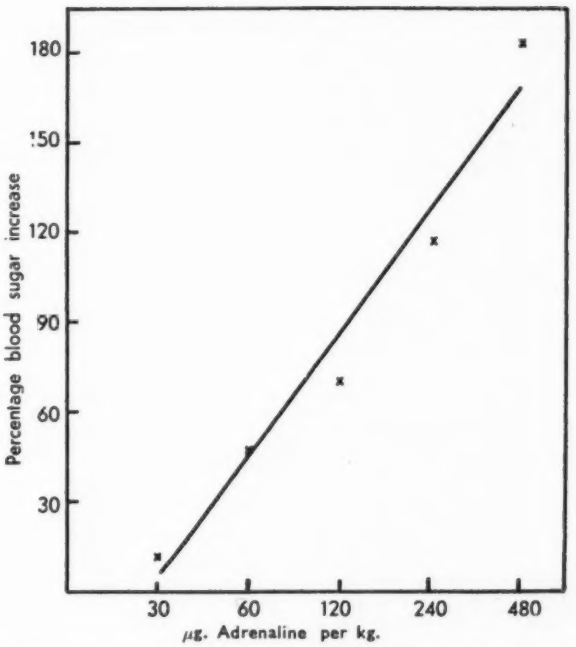

FIG. 1.—Dose response curve for hyperglycaemic action of adrenaline in 6 normal rabbits. Each cross represents mean response of all rabbits.

TABLE II

HYPERGLYCAEMIC RESPONSE TO 60 μg./kg. ADRENALINE AND TO 250 μg./kg. NORADRENALINE BEFORE AND AFTER 14 DAYS OF THYROID FEEDING

The response is expressed as the percentage blood sugar increase

Rabbit	Adrenaline			Noradrenaline		
	Before	After	Difference	Before	After	Difference
7	19·0	33·6	+14·6	26·6	20·6	−6·0
8	39·7	61·5	+21·8	36·0	42·4	+6·4
9	34·1	93·1	+59·0	51·5*	58·4	+6·9
10	20·1*	83·8	+63·7	29·3*	19·6	−9·7
11	110·6*	117·3	+6·7	97·4*	96·9	−0·5
12	79·0*	146·2	+67·2	63·6*	49·5	−14·1
13	145·3*	134·3	−11·0	105·0*	73·2	−31·8
14	79·6*	96·5	+16·9	39·0*	63·8	+24·8
15	73·4*	92·3	+18·9	28·2*	21·0	−7·2
16	78·4	86·5	+8·1	32·2	30·7	−1·5
17	44·4	63·2	+18·8	51·1*	21·8	−29·3
18	64·2	101·6	+37·4	68·2*	38·8	−29·4
19	93·2	112·8	+19·6	46·3*	22·3	−24·0
20	39·8	56·3	+16·5	25·7*		
21				35·3*	37·2	+1·9
22				24·9*	18·7	−6·2
23				25·6	21·7	−3·9
24				22·8	26·0	+3·2
25				34·2*	19·7	−14·5
Mean	65·8	91·4	+25·6	45·4	37·9	−7·5

* Mean of two experiments.

adrenaline was a blood-sugar rise of 65.8%, and that of the injection of noradrenaline was a blood-sugar rise of 50%. The difference between these two effects was 15.8%. Since an increase of 41.3% was produced by doubling the dose, it follows that the logarithm of the difference in potency between the doses of adrenaline and noradrenaline used was $\frac{15.8}{41.3} \times \log 2$, which is 0.115%. Hence the difference in potency between the doses of adrenaline and noradrenaline used was 1.3. Thus 1 μg. adrenaline was equal to 5.43 μg. noradrenaline in hyperglycaemic action.

Effect of Thyroid Feeding.—The effect of administering dried thyroid for 14 days is shown in Table II, where the change in the hyperglycaemic response is shown to 60 μg./kg. adrenaline in 14 rabbits, and in the hyperglycaemic response to 250 μg./kg. noradrenaline in 18 rabbits. The hyperglycaemic response to adrenaline increased in 13 out of the 14 rabbits, while that to noradrenaline increased in 5 out of 18 and decreased in 13 out of 18. The mean change in the hyperglycaemic response to adrenaline was an increase in the blood-sugar rise of 25.6%, so that the effect of 60 μg./kg. adrenaline in the rabbits after thyroid feeding was equivalent to that of 85 μg./kg. in the same rabbits before. The mean change in the hyperglycaemic response to noradrenaline was a decrease of 7.5% in the blood-sugar rise. Thus 250 μg./kg. noradrenaline had an effect in the rabbits after thyroid feeding equivalent to 220 μg./kg. in the same rabbits before. These changes in sensitivity to adrenaline and to noradrenaline were both significant (for adrenaline $P<0.01$; for noradrenaline $P<0.05$).

The experiments thus showed that the difference in the hyperglycaemic action of adrenaline and of noradrenaline was exaggerated and not diminished by thyroid feeding for 14 days. Whereas in the control observations the mean ratio for equiactive amounts of adrenaline and noradrenaline was 1 to 5.44 ± 1.12, in the thyroid-fed animals the mean ratio was 1 to 9.78 ± 1.13.

The mean loss of weight due to thyroid feeding was 270 g., and did not exceed 500 g. in any one rabbit. There was no correlation between the loss of weight and the increase in sensitivity to adrenaline.

Changes During Thyroid Feeding.—When thyroid feeding was prolonged beyond two weeks Burn and Marks (1925) found that the hyperglycaemic response to adrenaline decreased ; this change appeared to be due to the exhaustion of the liver glycogen reserve, as described by Cramer and Krause (1913).

Thus there were two phases in the effect of thyroid feeding on adrenaline hyperglycaemia, and it seemed probable that there were also two phases in the effect on noradrenaline hyperglycaemia. The change from one phase to the other might not coincide for the two substances, and the fall in the hyperglycaemic response to noradrenaline on the fourteenth day of thyroid feeding might have been preceded by a rise at an earlier stage. Experiments were therefore done to determine the responses to 250 μg./kg. noradrenaline on the third, seventh, and eleventh days of thyroid feeding. The results are given in Table III. They

TABLE III

HYPERGLYCAEMIC ACTION OF NORADRENALINE EXPRESSED AS PERCENTAGE BLOOD SUGAR INCREASE ON SUCCESSIVE DAYS OF THYROID FEEDING

Rabbit	Days of Thyroid Feeding				
	0	3	7	11	14
16	32·2	82·4	51·6	28·4	30·7
17	51·1*	35·8	38·7	25·6	21·8
18	68·2*	75·6	67·7	48·4	38·8
19	46·3*	64·8	79·6	86·0	22·3
20	25·7*	33·9	33·6	34·2	0
24	22·8	45·2	38·3	26·0	0
Mean	41·1	56·3	51·6	41·5	28·4

* Mean of two experiments.

show that the hyperglycaemia in five out of the six rabbits was increased during the first week and decreased in the second. Only one rabbit (No. 17) showed a decreased sensitivity to noradrenaline throughout the experiment. The individual maximal responses were observed between the third and the eleventh day, and Fig. 2 shows that the maximal mean effect was obtained on the third day, the difference from the controls being +15.2%.

In another group of four rabbits tested with 250 μg./kg. noradrenaline on the third day only, three showed an increased response, while one was less sensitive than before thyroid-feeding. The mean increase of response of all ten rabbits on the third day of thyroid-feeding was 14.3% ; this was significantly different from the controls ($P<0.05$). Thus the decrease in sensitivity to noradrenaline observed on the fourteenth day of thyroid-feeding was preceded by a significant increase in sensitivity during the first week.

To compare the change in sensitivity to noradrenaline with that to adrenaline, five out of the six rabbits in Table III were given 60 μg./kg. adrenaline on the seventh and fourteenth day. The

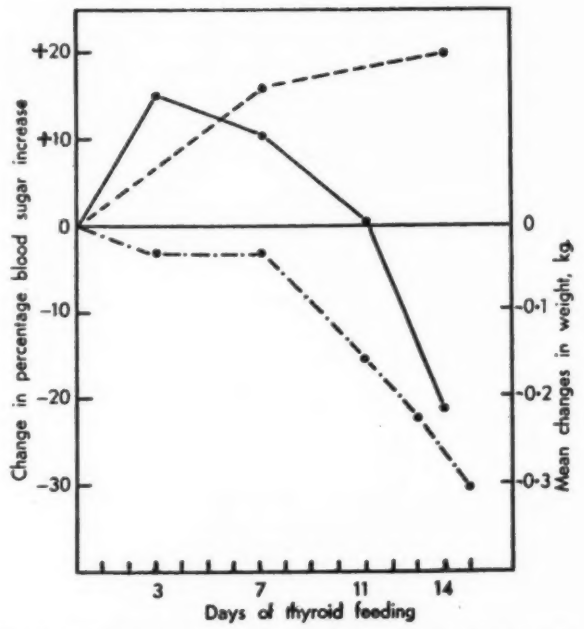

FIG. 2.—Changes in percentage blood sugar response to adrenaline and noradrenaline during thyroid feeding. Mean of controls taken as 0. The mean response of 6 rabbits to 250 μg./kg. noradrenaline (solid line) was increased during the first week and decreased during the second. The mean response of 5 of these 6 rabbits to 60 μg./kg. adrenaline (broken line) increased gradually. The loss of weight (dot–dash) appeared during the second week.

mean responses gradually increased, as shown in Fig. 2.

From the seventh day onwards the rabbits began to lose weight (Fig. 2). The onset of loss in weight appeared to coincide with the decrease in sensitivity to noradrenaline.

Relationship of Initial Blood Sugar to Hyperglycaemic Response.—Hemmingsen and Marks (1932) showed that the percentage blood-sugar reduction after insulin increased with increasing initial value of the blood sugar. The opposite was found to be true for the adrenaline and noradrenaline hyperglycaemia. The higher the initial blood-sugar value, the lower was the response to a given dose. A correlation between the initial blood sugar and the response to 60 μg./kg. adrenaline was observed in 41 experiments in normal rabbits, and was found to be highly significant ($P<0.001$), the regression coefficient being 1.838 ± 0.418. This meant that the magnitude of a response to a given dose of adrenaline depended on the initial blood-sugar value. The same relationship was observed for 250 μg./kg. noradrenaline and 30 μg./kg. adrenaline. In Fig. 3 the control rabbits were arranged in different groups according to their initial blood-sugar values, and the mean responses to 30 and 60 μg./kg. adrenaline and to 250 μg./kg. noradrenaline were plotted. The resulting curves were not parallel, the slope being steeper for 60 μg./kg. adrenaline than for 30 μg./kg. adrenaline. 250 μg./kg. noradrenaline, which had an intermediate action, showed an intermediate slope. Thus it was concluded that the slope of the curves did not depend on the nature of the substance injected but on its mean effect.

Thyroid-feeding seemed to affect this relationship. As adrenaline was more effective after thyroid-feeding the curve representing the response

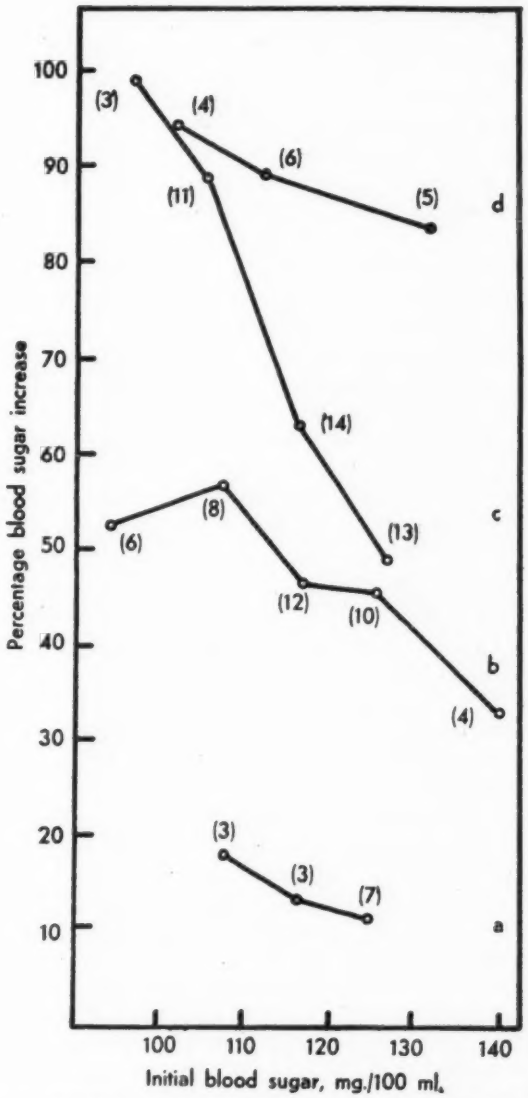

FIG. 3.—Relationship between initial blood sugar value and hyperglycaemic response to (*a*) 30 μg./kg. adrenaline, (*b*) 250 μg./kg. noradrenaline, and (*c*) 60 μg./kg. adrenaline in normal rabbits, and (*d*) to 60 μg./kg. adrenaline after 14 days of thyroid feeding. Numbers in brackets denote number of estimations made. For further details see text.

to 60 μg./kg. adrenaline after thyroid-feeding was expected to be steeper than that of the controls. It was, however, nearly horizontal (Fig. 3*d*), so that there was no correlation between initial blood-sugar value and response after thyroid-feeding. As this curve represented only a limited number of experiments, the difference between its slope and the slope of that for the controls (Fig. 3*c*) was not significant.

These results showed that a comparison of responses without consideration of the initial blood-sugar value might lead to error. Care was therefore taken to ensure that the mean responses calculated in Tables I–IV referred to approximately similar mean initial blood-sugar values. Thus, for instance, the mean initial blood-sugar value observed in the experiments with adrenaline before thyroid-feeding was 115.5 mg./100 ml. and after thyroid-feeding was 114.2 mg./100 ml. The corresponding values for the responses to noradrenaline were 115.5 mg./100 ml. and 114 mg./100 ml. These differences were too small to affect the results shown in Table II.

Changes in Amine Oxidase Activity of the Liver after Thyroid-feeding.—The determination of the amine oxidase activity of the livers of 14 control and 20 thyroid-fed rabbits confirmed the results of Spinks and Burn (1952), since amine oxidase activity fell from 96.5±3.68 μl. O_2/100 mg. liver/15 minutes in the controls, to 86.55±3.09 μl. O_2/100 mg. liver/15 minutes after thyroid-feeding. Thus by thyroid-feeding the amine oxidase activity of the liver was reduced to 89.6% of the value for the controls, a small but significant fall ($P<0.05$).

The Action of Ephedrine on Adrenaline Hyperglycaemia.—Since ephedrine is known to inhibit the action of amine oxidase (Blaschko, Richter, and Schlossmann, 1937), ephedrine should augment the hyperglycaemic action of adrenaline, if this is limited by the amine oxidase present. Ellis (1951) found that ephedrine in amounts as great as 25 mg./kg. did not alter the blood-sugar level of the rat, and Riechert and Schmieder (1941) found no change during three hours after subcutaneous injection of 10 mg. ephedrine into rabbits.

In a series of experiments on nine rabbits a single dose of 1 mg./kg. ephedrine was given subcutaneously 15 minutes before giving 60 μg./kg. adrenaline. No difference between the response to adrenaline and to adrenaline plus ephedrine was observed.

As the effect of this single dose of ephedrine probably did not last for more than one hour, a second series of 12 rabbits was tested in a different way: 1 mg./kg. ephedrine was given subcutaneously 15 minutes prior to 60 μg./kg. adrenaline, and the same dose of ephedrine was repeated 60, 120, and 180 minutes afterwards. As Table IV shows, the combination of adrenaline and ephedrine in this series was more effective than adrenaline alone in 11 of 12 rabbits. The mean increase in response was +31.7% ($P<0.02$), the mean initial blood-sugar value being approximately the same in both groups. Thus when a certain ephedrine level was maintained for more than three hours the effect of adrenaline was significantly potentiated. In the presence of ephedrine, 60 μg./kg. adrenaline had the same hyperglycaemic effect as 96 μg./kg. alone. This potentiation can be compared with the effect of thyroid-feeding, after which 60 μg./kg. adrenaline had the same effect as 85 μg./kg. before.

TABLE IV

THE POTENTIATION OF THE HYPERGLYCAEMIC ACTION OF ADRENALINE BY EPHEDRINE

Figures are percentage blood sugar rise

Rabbit No.	Adrenaline	Adrenaline plus Ephedrine	Difference
26	59·5*	114·9	+55·4
27	58·6	74·3*	+15·7
28	40·7*	164·9	+124·2
29	62·0	101·7*	+39·7
30	91·0	45·2*	−45·8
31	100·8	120·2	+19·4
32	24·1	51·6	+27·5
33	53·8*	109·6	+55·8
34	49·6*	67·6	+18·0
35	61·9	98·5	+36·6
36	71·0	92·4	+21·4
37	45 0*	57·2	+12·2
Mean	59·8	91·5	+31·7

* Mean of two experiments.

The amine oxidase activity of the 12 rabbit livers of the experiments of Table IV was determined and a calculation was made to see whether there was any correlation between amine oxidase activity and (*a*) the response to the test dose of adrenaline, (*b*) the potentiating effect of ephedrine. No correlation was, however, found.

The Ratio of Equi-effective Doses of Adrenaline and Noradrenaline on the Isolated Rabbit Duodenum Before and After Thyroid-feeding.—To find if thyroid-feeding affected the ratio of equiactive amounts of adrenaline and noradrenaline on the isolated rabbit duodenum in the same way as it affected the ratio of doses producing the same hyperglycaemia, the ratio was determined on the isolated duodenum of six control and six thyroid-fed rabbits. Table V shows that it did not change, and that the mean ratio agreed with

TABLE V

RATIO OF EQUIACTIVE AMOUNTS OF ADRENALINE AND NORADRENALINE ON ISOLATED STRIPS OF DUODENUM TAKEN FROM CONTROL RABBITS AND FROM RABBITS AFTER 14 DAYS OF THYROID-FEEDING

Contro's	After 14 Days of Thyroid-feeding
1 : 1·11	1 : 0·85
1 : 0·6	1 : 0·9
1 : 0·9	1 : 4·0
1 : 1·1	1 : 1·0
1 : 1·3	1 : 1·5
1 : 1·25	1 : 0·42
Mean 1 : 1·01	1 : 1·11

the 1:1 value found by West (1947) and by Luduena, Ananenko, Siegmund, and Miller (1949). The minimal effective doses were not tested, but adrenaline and noradrenaline appeared to be more effective after thyroid-feeding, since a comparable relaxation of the intestine was obtained with about one-third of the normal dose.

DISCUSSION

The hyperglycaemia produced in rabbits by the subcutaneous injection of adrenaline is generally accepted as being a direct action of adrenaline on the liver. Miculicich (1912) showed that it was absent after the injection of ergotoxine, and this was confirmed by Burn (1915). Previous statements that thyroid-feeding increases adrenaline hyperglycaemia have now been confirmed, by determining a dose-response curve for adrenaline, and then using a large group of rabbits for a study of the effect of thyroid-feeding. The administration of 0.2 g. thyroid daily augmented the hyperglycaemic response to adrenaline, so that at the end of two weeks the response to 60 μg./kg. was equivalent to the response to 85 μg./kg. initially.

One explanation of this increased effect would be that thyroid-feeding led to a fall in the enzyme which destroys adrenaline. While the steps in the breakdown of adrenaline are not yet generally agreed, there is increasing evidence that amine oxidase is concerned. The work of Schayer and his colleagues (see Schayer and Smiley, 1953) on the fate of C^{14} labelled epinephrine has led them to the conclusion that amine oxidase plays a major role in epinephrine metabolism in the intact animal. Amine oxidase is present in the liver, and Spinks and Burn (1952) found that in rabbits fed with thyroid extract for 14 days there was a significant fall in the amine oxidase in the liver. Their observations have been confirmed in these experiments, and this suggests that the increase in adrenaline hyperglycaemia may indeed be due to the decline in the amount of the enzyme. Further support was gained for this view by finding that ephedrine potentiated adrenaline hyperglycaemia when 1 mg. was given before adrenaline and at hourly intervals after. This agreed with Gaddum and Kwiatkowski's findings in blood vessels, nictitating membrane, and frog heart (1938).

The mean potency of noradrenaline in raising the blood sugar was found to be 18% of that of adrenaline. The effect of thyroid-feeding was to increase its mean hyperglycaemic action more rapidly than that of adrenaline during the first three days; but by seven days the increase in the adrenaline effect was the greater, and by two weeks the noradrenaline effect had declined below the initial value. This result did not support the expectation, outlined earlier, that thyroid-feeding would augment noradrenaline hyperglycaemia more than it augmented adrenaline hyperglycaemia. It pointed to a difference in the action of the two substances. While adrenaline breaks down muscle glycogen to form lactic acid which is then converted to liver glycogen in the lactic acid cycle (see Soskin, 1941), noradrenaline does not have this action (Bearn, Billing, and Sherlock, 1951; Russell, personal communication). The work of Geiger and Schmidt (1928) showed that the breakdown of muscle glycogen can play a part in the hyperglycaemic effect of adrenaline. They treated dogs for several days with phloridzin until the liver glycogen was greatly diminished. They found that adrenaline was still able to produce a rise of blood sugar, this being accompanied by a complete loss of muscle glycogen. Thus, while the blood-sugar response to noradrenaline depends on the liver glycogen only, that of adrenaline probably depends on muscle glycogen as well.

How great a fall in amine oxidase would be required to affect the adrenaline response is unknown. Evidence was obtained by Nachmansohn (1952) that conduction in nerve ceased only when almost the whole of the cholinesterase present was inhibited, and that the inhibition of a small proportion of the cholinesterase had no effect on function. Observations in other tissues show that this is not a general rule. A concentration of eserine 4×10^{-8} g./ml. causes a prompt increase in the tone of a loop of rabbit duodenum (Burn, 1952b) and a concentration of eserine 10^{-8} causes the rate of isolated rabbit auricles to become slower (Burn and Kottegoda, 1953). *In vitro* experiments by Miss H. Shelley have shown that when ACh is used as a substrate the cholinesterase in the auricles was inhibited 15.4% by eserine 3×10^{-8} g./ml. and was not appreciably affected by half this concentration.

Another explanation for the hypersensitivity to adrenaline produced by thyroid feeding must be mentioned. The observed relationship of initial blood sugar and response to a given dose of adrenaline may be due to two or more factors. The utilization of glucose by extra hepatic tissue has been found to increase greatly with a rise in the blood sugar level. On the other hand, the liver maintains the blood sugar at a normal level by increasing the intake and decreasing the output of glucose (Soskin, 1941). These two different regulating mechanisms may well account for the smaller response to a given dose of adrenaline at a high initial value.

The fact that after thyroid feeding the response to adrenaline does not depend on the initial blood sugar suggests an impairment of one or both of these regulating mechanisms. The effect of thyroid hormone on the utilization of glucose by extra hepatic tissues is unknown, while both the anterior pituitary hormones and insulin control the maintenance of the normal blood sugar level by the liver (Soskin, 1941). As thyroid hormone affects both the anterior pituitary and the β cells of the pancreas (Houssay, Foglia, and Martinez, 1946), it is possible that the increased adrenaline hyperglycaemia is due to changes in the mechanisms which control the blood sugar.

Summary

1. A dose-response curve has been determined for the hyperglycaemic action of adrenaline in normal rabbits. The response bore a linear relation to the log dose.

2. The dose-response curve for noradrenaline was parallel to that for adrenaline; noradrenaline was found to have 18% of the action of adrenaline.

3. The mean hyperglycaemic response to 60 μg./kg. adrenaline was increased by feeding 0.2 g. thyroid daily for 14 days so that it became equal to the response to 85 μg./kg. before thyroid feeding.

4. The mean hyperglycaemic response to 250 μg./kg. noradrenaline was increased by feeding 0.2 g. thyroid daily during the first three days to a greater extent than was the adrenaline response. Thereafter the noradrenaline response diminished, and at the end of 14 days was less than the initial response.

5. The response to adrenaline in normal rabbits was found to depend on the height of the resting blood sugar. In thyroid-fed rabbits the response to adrenaline was independent of the height of the resting blood sugar.

6. Ephedrine potentiated adrenaline hyperglycaemia to about the same extent as did thyroid feeding when 1 mg. was injected 15 min. before the adrenaline and at hourly intervals for 3 hr. after.

7. Thyroid feeding produced a fall of liver amine oxidase to 89.6% of the control value, this fall being significant, and approximately the same as that previously found.

I am deeply indebted to Professor J. H. Burn for his guidance and encouragement throughout this investigation.

The experiments were carried out during the tenure of a British Council Scholarship.

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THE PROTECTIVE ACTION OF ANTIHISTAMINIC AND SYMPATHOMIMETIC AEROSOLS IN ANAPHYLACTIC MICROSHOCK OF THE GUINEA-PIG

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(RECEIVED JULY 13, 1953)

In two earlier papers we have described the protective effect of varying amounts of a number of antihistamines (Armitage, Herxheimer, and Rosa, 1952) and sympathomimetic amines (Herxheimer and Rosa, 1953) given by injection. This paper deals with experiments in which some of these substances were given by aerosol. In some other experiments the duration of the protective effect by injection was investigated in order to compare it with that following aerosol inhalation.

METHOD

Our method as previously described (Herxheimer, 1952a ; Armitage *et al.*, 1952) was modified. Instead of the protecting substance being injected, usually given one hour before exposure to the shocking agent, the animals inhaled it in an aerosol. The dosage of protecting substance was varied by altering either its concentration or the period of inhalation. The test substances were dissolved in a mixture of equal parts of water and propylene glycol. The aerosolization of the protective substances was produced by compressed air from a cylinder, the air pressure being controlled by a flowmeter and kept constant. In a few experiments a commercial nebulizer was used, but in most a "midget scrubber" as described by Dautrebande (1951). These two instruments differ in that the droplets produced by the commercial nebulizer are larger, probably of a diameter between 1 and 3 μ, while those produced by the midget scrubber are, according to Dautrebande, of more uniform size, with a diameter well below 1 μ. The smaller droplets penetrate further into the lungs, but adhere less easily to the walls of the air passages. In addition their total weight, and therefore their action, is smaller than that of the larger particles. Though an exact comparison of both methods of nebulization is difficult, we have tried to compare them clinically by measuring the effect of isoprenaline aerosols in mild induced attacks of asthma. Such a comparison showed that the aerosol with uniform droplet size below 1 μ diameter is the less effective.

The percentage retention of a drug inhaled as an aerosol will depend partly on the amount of the drug inhaled. How much is inhaled will depend on the aerosol density in the aerosol chamber, the respiratory volume, and the dead space. Other factors include the aerosol density in the air passages, the size of the particles, their electrical charge, and the nature of the inhaled substance.

Accurate data about the retention of aerosol in the lungs are lacking. Abramson, Reiter, Gettner, and Sklarofsky (1949) have found in experiments with a phenolsulphonphthalein aerosol in man that not more than one-tenth of the inhaled amount was retained. According to Dautrebande (1951) the output of the midget scrubber nebulizer at 8 lb./sq. in.—the pressure we have applied—is 3 ml./hr. This equals 30 mg./hr. or 0.5 mg./min. of the substance used, if it is in 1% solution. As the output of air by this instrument is 5 l./min. at the same pressure, the concentration of aerosol in the chamber will be approximately 1 mg./10 l.

According to Ainsworth (1953), to whom I am obliged for these data, the tidal air of a guinea-pig of 300–500 g. weight varies from 2–3 c.c., and the respiratory volume from 240–400 c.c./min. If these data are used as the basis of a very approximate calculation, the animal would inhale about 333 c.c./min. of the aerosol containing 1 mg. of the drug in 10 l. of air, or about 0.033 mg./min. It would retain one-tenth of this, namely about 3 μg. in one min., or 0.5 μg. in 10 sec. This calculated amount is probably a little too great, as the respiration of the guinea-pig is very shallow (resp. rate 110–144/min.) and the proportion of dead space ventilation in the respiratory minute volume therefore high. Our calculation shows that the amounts of drug retained during short periods must be very small indeed. They are smaller than the lowest doses of sympathomimetic or antihistaminic substances which gave definite protection by intramuscular injection (Armitage *et al.*, 1952 ; Herxheimer and Rosa, 1953).

The guinea-pigs were sensitized with egg albumin as in our previous experiments. Shock exposure was always by aerosol through the same commercial nebulizer at the same pressure.

Before a typical experiment the aerosol chamber is filled with the protecting aerosol. Some animals are then transferred into the chamber and left there, the

aerosol flowing through the chamber at a constant rate for the period intended. At the end of this period the chamber is lifted off its base and quickly ventilated, the animals removed, the chamber replaced and filled with albumin aerosol for the shock experiment. The animals are then put back into the chamber and the preconvulsion time is determined. In each animal the "percentage protection" index was calculated by the modified method described by Herxheimer and Rosa (1953).

The substances used were tripelennamine, promethazine, diphenhydramine, chlorcyclizine, isoprenaline, adrenaline, noradrenaline, ephedrine, and aminophylline. Detailed dose-response relationships were investigated only for one antihistamine (tripelennamine), one sympathomimetic amine (isoprenaline), and for aminophylline. The duration of the protective effect after injection was investigated with promethazine, diphenhydramine, chlorcyclizine, and aminophylline.

Results

The experiments with tripelennamine (Table I) show that aerosol inhalation for only one minute confers a definite, though not very strong, protection. Inhalation of a 1% solution for 3 min. gives about as strong a protection as an intramuscular injection of 1.0 or 3.0 mg./kg. No greater protection is given if the 1% solution is replaced by the 2% solution, or if the inhalation period is extended to 15 min.

The protection thus achieved is of short duration. One hour later it had decreased, often considerably, whatever the period of inhalation. Two hours later there was no longer evidence of protection in two out of three experiments. In the third, protection was still present but very weak. By contrast, after injection of 1 mg. or 3 mg./kg. a significant degree of protection was still present 4 and 6 hours later (Table I).

Diphenhydramine, chlorcyclizine, and promethazine (Table I) were examined in less detail. A 2% diphenhydramine aerosol gave no significant protection when inhaled for 5 or 10 min. If this period was extended to 15 or 30 min., the protection given was pronounced, about the same as that estimated for intramuscular injection of 2.0 mg./kg. and that obtained for 6.0 mg./kg. Two hours after inhalation no significant protection was present. When 6 mg. was injected intramuscularly, protection was still at its maximum after 6 hr. but had decreased sharply after 8 and 12 hr. After the injection of 2 mg./kg. no protection could be found after 8 hr.

Inhalation of 0.5% promethazine gave no protection after 2 min., but good protection after 5 min. The 3% solution gave a very high protection after inhalation for 10 min. When the animals were exposed for 30 min., they became dyspnoeic whilst in the promethazine aerosol. The same happened to two out of five animals breathing a 10% aerosol for 15 min. The duration of this protection was not followed up, but that following intramuscular injection was found to remain at or near its maximum for at least 12 hr. After 17 and 22½ hr. it was weak but still present.

TABLE I

EFFECT OF ANTIHISTAMINIC DRUGS ON ANAPHYLACTIC MICROSHOCK

Substance and Route of Administration	Conc. Inhaled (%)	Duration of Inhalation (min.)	Dose Injected (mg./kg.)	No. of Animals	% Protection ± Standard Error	Interval between Drug and Exposure to Shock (min.)
Tripelennamine						
(*a*) Aerosol	1	1		5	40·4 ± 3·7*	0
	1	1		5	24·8 ± 10·8	0
	1	3		4	59·0 ± 9·2*	0
				3	60·0 ± 1·2*	0
	1	5		3	64·0 ± 3·5*	0
				3	68·7 ± 6·3*	0
	2	5		3	78·0 ± 4·4*	0
	2	15		5	63·0 ± 10·9*	0
	1	5		6	52·7 ± 3·9*	60
	1	5		6	10·7 ± 8·9	120
	2	5		6	34·0 ± 8·0*	60
	2	5		6	25·3 ± 8·9*	120
	2	15		7	48·9 ± 10·9*	60
	2	15		6	8·3 ± 10·1	120
	2	15		5	23·0 ± 10·5	300
(*b*) Injection			1·0	5	62·2 ± 8·2*	60
			1·0	6	25·0 ± 8·7*	360
			3·0	5	54·2 ± 8·4*	60
			3·0	5	59·8 ± 2·9*	240
Diphenhydramine						
(*a*) Aerosol	2	5		6	31·5 ± 13·4	0
	2	10		5	20·2 ± 17·4	0
	2	15		6	59·3 ± 6·9*	0
	2	30		7	45·4 ± 10·1*	0
	2	15		5	− 6·6 ± 14·7	120
(*b*) Injection			0·9	7	33·3 ± 3·2*	60
			2·0[1]	6	17·3 ± 11·5[3]	60
			6·0[1]	6	44·7 ± 9·7*	60
			2·0	6	−11·3 ± 8·4	480
			6·0	4	67·0 ± 7·5*	360
			6·0	5	37·2 ± 9·8*	480
			6·0	6	32·3 ± 7·0*	540
Promethazine						
(*a*) Aerosol	0·5	2		7	17·9 ± 14·5	0
	0·5	5		6	51·3 ± 4·3*	0
	3	10		5	66·2 ± 4·9*	0
	10	15		2	69·0 ± 7·0	0
(*b*) Injection			0·75[1]	6	68·8 ± 7·4*	60
			0·75[1]	7	75·6 ± 5·1*	60
			1·5[1]	7	66·0 ± 6·7*	60
			1·0	5	75·2 ± 6·6*	390
			1·0	5	70·0 ± 4·6*	510
			1·0	6	56·7 ± 7·0*	540
			1·0	6	21·2 ± 10·1	1,020
			1·0	6	20·8 ± 6·1*	1,350
Chlorcyclizine						
(*a*) Aerosol	2	5		5	1·6 ± 11·9	0
	2	15		3	3·3 ± 12·7	0
	5	15		5	69·0 ± 7·5[2]*	0
(*b*) Injection			6·0[1]	5	58·2 ± 2·4*	60
			6·0	6	30·7 ± 11·1*	480
			6·0	4	50·0 ± 8·2*	720

* Statistically significant protection:
[1] Experiment taken from earlier work (Armitage *et al.*, 1952).
[2] All animals dyspnoeic in chlorcyclizine aerosol.
[3] Estimated protection 44·9.

Inhalation of a 2% chlorcyclizine solution gave no protection. If a 5% solution was used, all animals became slightly dyspnoeic but were nevertheless well protected. After intramuscular injection of 6 mg. chlorcyclizine a moderate protection was observed after 8 and 12 hours.

The protection given by 90 to 105 sec. of 1% isoprenaline (Table II) was strong, but it disappeared almost completely after 30 or 60 min. Even inhalations as short as 10 and 15 sec. gave a mild but definite protection. The experiments with 1% adrenaline (Table II) gave similar results. The effect of noradrenaline was weaker (Table II).

Ephedrine hydrochloride 1% given for 15 min. caused dyspnoea. The ½% aerosol had no such effect and gave a limited protection.

The experiments with aminophylline (Table III) had no clear result. By contrast with the antihistamines no dyspnoea occurred with the higher concentration used (12½%), but the protection given even by long exposures was slight or absent. The lower concentrations (1%–5%) seemed to give the same or even a better protection than the higher ones regardless of the period of exposure. In no instance did the percentage protection reach the maximum values seen with tripelennamine, adrenaline and isoprenaline.

The duration of the effect of the isoprenaline and adrenaline (Table II) aerosols was extremely short; the isoprenaline effect had disappeared after 30 min. and that of adrenaline after 1 hr. The injection of both substances (Table II) was effective for several hours. Aminophylline injections (Table III), which gave complete immediate protection, lost their effect 6 hr. later.

TABLE II

EFFECT OF SYMPATHOMIMETIC DRUGS ON ANAPHYLACTIC MICROSHOCK

Substance and Route of Administration	Conc. inhaled (%)	Duration of Inhalation (sec.)	Dose Injected (mg./kg.)	No. of Animals	% Protection ± Standard Error	Interval between Drug and Exposure to Shock (min.)
Isoprenaline						
(*a*) Aerosol	1	10		5	28·0±12·2	0
	1	10		4	33·3± 6·8*	0
	1	15		5	36·4±10·9*	0
	1	30		5	45·2± 7·0*	0
	1	30+15[1]		2	68·0	0
	1	90+15[1]		3	85·7± 7·3*	0
	1	90+15[1]		9	1·9± 4·7	30
	1	90+15[1]		6	17·5±12·8	60
(*b*) Injection			0·1	3[2]	85·3±14·7*	15
			0·1	7[2]	68·4± 5·0*	15
			0·1	3	83·7± 1·8*	30
			0·1	5	72·2± 9·2*	60
			0·1	3	44·7± 4·1*	180
			0·1	3	30·3±15·9	360
Adrenaline						
(*a*) Aerosol	1	10		5	53·0± 9·6*	0
	1	15		5	69·7± 6·0*	0
	1	20		4	65·0±13·7*	0
	1	60		5	79·2± 1·4*	0
	1	90+15[1]		6	47·3± 2·1*	30
	1	90+15[1]		4	40·5± 6·6*	30
	1	90+15[1]		4	13·8± 7·6	60
(*b*) Injection			0·2	7[2]	57·1± 5·8*	15
			0·2	4	35·0± 9·0*	60
			0·2	6	66·8± 4·4*	60
			0·2	4	52·5± 6·4*	120
			0·2	3	11·7± 7·5	180
			0·2	3	71·6±23·5	180
			0·2	5	54·4±21·6	240
Noradrenaline						
Aerosol	1	20		5	52·2± 5·2*	0
	1	20		4	50·5± 7·1*	0
	1	60		5	41·4± 8·4*	0
	1	60		6	52·2± 8·1*	0
	1	120		3	75·0± 5·8*	0

* Statistically significant protection.

[1] After 90 sec. exposure the inflow of aerosol was stopped and the animals allowed to remain 15 sec. longer in the aerosol chamber. In the other experiments they were removed immediately.

[2] Experiment taken from earlier work (Herxheimer and Rosa, 1953).

TABLE III

EFFECT OF AMINOPHYLLINE ON ANAPHYLACTIC MICROSHOCK

Route of Administration	Conc. Inhaled (%)	Duration of Inhalation (min.)	Dose Injected (mg./kg.)	No. of Animals	% Protection ± Standard Error	Interval between Drug and Exposure to Shock (min.)
Aerosol ..	1	10		5	35·0± 8·3*	0
	1	60		5	50·6± 8·2*	0
	2	10		4	43·0± 9·0*	0
	2	30		4	41·0± 5·7*	0
	5	3		3	11·7±10·9	0
	5	5		3	10·3±11·7	0
	5	10		5	47·2± 8·0*	0
	5	15		3	41·3± 2·8*	0
	5	20		4	50·8± 2·3*	0
	5	20		4	5·.·3±12·8*	0
	5	60		3	22·3± 1·8*	
	12·5	10		2	16·5±18·5	0
	12·5	20		5	18·5±13·1	0
	12·5	20		2	10·5± 0·5	0
	12·5	30		5	48·6± 9·6*	0
	12.5	60		4	2·.0±10·4	0
Injection			100	4[1]	100·0*	20
			100	6	83·0± 3·0*	60
			100	4	60±22·0	300
			100	5	37·4±17·6	360

* Statistically significant protection.

[1] Experiment taken from earlier work (Herxheimer and Rosa, 1953).

DISCUSSION

The high degree of protection achieved by inhalation may seem surprising. It is certain that the amounts of substance inhaled and absorbed during an exposure of 10 sec. must be very small indeed. Moreover, the effects of the absorption of these small amounts appear within a few seconds. A similar observation has been made in man. In an attack of induced asthma the effect of 10 sec. isoprenaline inhalation becomes apparent about 20 sec. after the end of the inhalation, when the respiration becomes deeper and the rate increases. This appears to be due to a central stimulating effect on respiration. The

decrease of bronchial obstruction, measured by the speed of the expiratory flow or by the vital capacity, shows the effect of the drug at the same time and reaches its maximum after 1 min. or a little later. If the substance is taken perlingually, its effect does not become noticeable for 3 or 4 min., and the amount needed is many times greater. The longer period of time is needed for the absorption of the substance and its transport by the blood stream to its site of action. By inhalation the substances are effective almost immediately, because only a negligible interval is required for absorption and transport. We are thus driven to the conclusion that the substances which act with such speed act in the cells in which they are absorbed. The few seconds which pass before their action occurs are probably required for the penetration of the film of mucus covering the cells of the mucous membrane. This would explain how an inhalation of 1% adrenaline for about 15 sec., which could not lead to an absorption of more than a few micrograms, could achieve as great a degree of protection as the injection of 200 μg. The latter amount is distributed throughout the body in order to reach the bronchi, and the amount acting on the mucous membrane can be only a fraction of the amount injected. The same is seen with tripelennamine. Here the inhalation of a 1% solution, with a probable retention of about 6 μg., gives a percentage protection of about 40, whilst by injection 50 μg. was the smallest dose with which any noticeable protection was achieved.

On the other hand, our assumption would also explain the short duration of the effect of inhalation. The small amount deposited in the mucous membrane would at once be destroyed or absorbed into the blood stream. In either event the effective concentration would be reduced considerably within a short time, and in fact the high protection given by isoprenaline and adrenaline disappears within about half an hour, whilst that given by the injection lasts for a few hours. This difference points to the fact that it is not so much destruction of the substance as its changing distribution which causes the difference in the duration of its effect. A few micrograms spread over the whole body constitute a subthreshold dose, whereas the same amount concentrated in the bronchial membrane has a demonstrable effect.

This becomes still more evident with the antihistamines. If injected, their effect lasts for many hours; in the case of promethazine it can be traced even after 17 and 22 hours; in others it is less, and, on the whole, the effective periods observed are very similar to those Bain (1949) has found for the action of these drugs against histamine. The effect of aerosols, however, disappears after 1–2 hours. Thus the effect of the antihistamines lasts longer than that of the sympathomimetic amines whether they are inhaled or injected, but for both groups the inhalation effect is much the shorter. It is interesting to note that similar differences in the effect of the aerosols of the various antihistamines are observed whether these are injected or inhaled. In agreement with clinical experience tripelennamine and promethazine have been found stronger than diphenhydramine and chlorcyclizine.

Aminophylline differs from antihistaminic and sympathomimetic drugs in various respects. If injected, its protective effect is greater than with either of these groups; the duration of its effect is less than that of the antihistamines, but longer than that of the sympathomimetic amines. The effect of aminophylline aerosol is, by contrast, disappointing. Its protective action is weak, and it cannot be increased by longer inhalation even at higher concentration. This suggests that its mechanism of action differs from that of the other substances.

Little is known of the mechanism of aminophylline action in general, and any explanation is therefore bound to be purely speculative. The low level of protection by inhalation may suggest that the aerosol does not act upon the same cells on the surface of the mucosa as do the other substances. If aminophylline were to act in a deeper layer of the tissue—if it were to act, for example, on blood vessels other than the most superficial capillaries—it would require transport to this site of action. Its accumulation at the site of absorption would not increase its effect. Once its transport had begun it would pass its particular site of action in the deeper layers of the tissue, since it is only in transit. This would prevent its accumulation at this site, and the action would thus be produced by only a part of the inhaled total when that happened to pass the reactive tissue or organs.

A curious feature of these experiments is the effect of higher concentrations of some antihistamines which cause an asthma-like dyspnoea instead of protecting against it, as lower concentrations do. Similar reactions have been found in human asthma with promethazine, tripelennamine, and ephedrine (Herxheimer, 1952b). The reasons for this are not clear; it may be recalled that Arunlakshana has found (1953) that antihistamines under some conditions cause the release of histamine. Another possible reason is the local irritant

action of some antihistamines which Bain, Hellier, and Warin (1948) found when the drugs were injected intradermally.

The practical consequences of these results for the pharmacology of human asthma are mainly confirmatory. It has been known for a long time that adrenaline and isoprenaline aerosols have an almost immediate relieving effect in bronchial asthma, and that they are less likely to cause unpleasant systemic side effects than oral or subcutaneous application. What has been less well known is the short duration of this relieving effect by inhalation. There are many chronic asthmatics whose bronchial obstruction is continuous and does not fluctuate much in intensity. These patients often insist that they must inhale every 1½–3 hr., and some clinicians are inclined to attribute this insistence to habit rather than a necessity. Our experiments show that there may often be a real necessity for repeated inhalation.

The protective action of an antihistaminic aerosol has been noticed before (Herxheimer, 1949; Friebel, 1953). In practice, the relieving effect of sympathomimetic aerosols needs less time and is stronger than that of antihistaminic aerosols; the latter therefore are not recommended for the treatment of asthma.

The long duration of the injection effect of the antihistamines is also borne out by practical experience. Not only has Bain (1949), as mentioned before, found this long duration in their action against histamine itself, but in asthmatic patients this long lasting effect can be easily observed. If a patient is given, for instance, diphenhydramine at bedtime, its antiasthmatic (and its sedative) effect decreases sharply after 6–8 hr. Promethazine and chlorcyclizine, however, still show a definite effect in the morning, often 12–13 hr. or more after administration.

Summary

1. Antihistamines and sympathomimetic amines administered by aerosol protect guinea-pigs against anaphylactic shock. Relatively small amounts inhaled give a high degree of protection, often equalling that achieved by intramuscular injection. Whilst the effect of the latter lasts for many hours and then slowly decreases, that of the former decreases almost immediately. Protection by an aerosol is hardly detectable after 2 hr. with antihistamines and after 30 min. with sympathomimetic amines.

2. For approximately equal degrees of protection, much smaller total amounts are necessary by inhalation than by injection. Given by inhalation, the drugs are initially concentrated at their site of action, but are presumably quickly removed by the circulation. Given by injection, the drugs are widely distributed and must accordingly be given in much greater amount. Their action persists for a much longer period, until they are excreted or destroyed.

3. Aminophylline aerosol has a weak protective action against anaphylactic shock, and the protection cannot be increased by longer inhalation or by an increase in the concentration of the inhalant. By contrast, the protection given by intraperitoneal aminophylline is higher than that of any antihistaminic or sympathomimetic drug. It is suggested that the relative inefficiency of the aminophylline aerosol may be accounted for by aminophylline having a different site of action from that of the antihistaminic and sympathomimetic drugs.

4. The differences between the protective strength of the various antihistamines and their duration of action correspond to the differences observed clinically.

5. The effect of antihistaminic substances lasts longer than that of sympathomimetic amines whatever the route of administration.

This work was assisted by a personal grant from the Medical Research Council and by a grant for technical assistance from the Asthma Research Council. I am also indebted to Dr. P. Armitage, of the Statistical Research Unit, M.R.C., for the statistical analysis of the results.

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THE FAILURE OF RESPIRATION IN DEATH BY ANTICHOLINESTERASE POISONING

BY

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(RECEIVED AUGUST 21, 1953)

Many organic phosphorus compounds are now known which are potent inhibitors of cholinesterase, and all have high toxicities. A comprehensive list of such compounds has been published by Holmstedt (1951), and from this we can distinguish several substances which have proved to be biologically interesting and important, such as di*iso*propyl phosphofluoridate (DFP), diethyl *p*-nitrophenyl phosphate (E600; "Mintacol"), ethyl NN-dimethylphosphoramidocyanidate ("Tabun"), *iso*propyl methylphosphonofluoridate ("Sarin"), 3 : 3-dimethyl-*n*-butyl 2-methylphosphonofluoridate ("Soman"), and tetraethyl pyrophosphate (TEPP).

All these compounds produce qualitatively similar effects when administered to mammals. A lethal dose produces the following picture in the conscious animal. There is muscular fasciculation, followed by violent incoordinate convulsive movements, prostration, gasping respiratory movements and signs of "air hunger," engorgement of the veins, and often micturition and defaecation. Unconsciousness follows, respiration ceases, the heart slows and the pupils may contract (miosis always occurs early if the eyes are exposed directly to the drug). Then the skin capillaries collapse and finally the heart ceases to beat.

At autopsy the picture is similar whatever the method of administration. The diaphragm is elevated, with the lungs usually collapsed and ischaemic athough occasionally they are congested. There is spasm of the small intestine and the abdominal viscera, with peritoneal effusion, and the spanchnic veins are engorged with dark venous blood. The right heart is distended and the left ventricle is often empty. Apart from the "venous" colour of the arterial blood, the brain is generally normal in appearance, but there are sometimes a few petechiae in the brain substance.

The general picture in all the species studied—mouse, rat, guinea-pig, rabbit, cat, dog, monkey, sheep, and goat—is characteristic of the asphyxial state and it is evident that failure of respiration is the predominant effect produced by intoxication with an anticholinesterase (DFP, E600, tabun, sarin, soman, TEPP).

There is, however, no general agreement as to how the final respiratory failure occurs. Failure of the respiratory control mechanism in the central nervous system has been suggested as the dominant factor (Modell, Krop, Hitchcock, and Riker, 1946; Freedman and Himwich, 1949); others stress the part played by bronchospasm, stating that this by itself can prove fatal (Koppanyi, 1948); others again are of the opinion that neuromuscular paralysis may be a significant factor (Riker and Wescoe, 1949). The evidence for each of these three possible causes will be considered separately.

Whatever the cause of the respiratory failure may be, it usually leads, in absence of treatment, to death from asphyxia.

Treatment is along two lines—application of artificial respiration and administration of atropine. Conjointly, these are more effective than is either course separately. Although atropine is only able to counteract the muscarinic actions of acetylcholine its efficacy in restoring the respiratory centres to an adequate state of activity is unquestionable (Douglas and Matthews, 1952).

METHODS

Experiments were carried out on cats, dogs, rabbits, monkeys, sheep, goats, mice, rats, and guinea-pigs. Eserine, DFP, E600, tabun, sarin, soman, and TEPP were chiefly used. Administration has been most commonly by intravenous injection or by inhalation. For some purposes we have used subcutaneous, intramuscular, intra-arterial, and intracisternal injections;

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injections into special parts of the C.N.S. by microsyringe; instillations into the conjunctival sac, and applications to the clipped or depilated skin.

Phenobarbitone or pentobarbitone intraperitoneally, or urethane intravenously, were the anaesthetics most commonly used. Some cats were decapitated under ether and given artificial respiration.

Respiratory volumes were recorded by a water-floated counterpoised spirometer; diaphragm contractions by the phrenograph described by de Candole, Douglas, and Spencer (1950); intrapleural pressure by an intraoesophageal balloon, or by a pleural cannula; and intra-abdominal pressures by a balloon inserted at the midline. Arterial pressures were recorded from the femoral artery by a Hg manometer. Intravenous injections were made into the femoral vein.

Nerve action potentials were amplified by a five-stage AC coupled amplifier and were photographed from the cathode-ray tube face.

Other methods used are described in the appropriate parts of the text.

Results

The Production of Bronchoconstriction.—One salient feature of anticholinesterase poisoning in experimental animals is a lessened inflatability of the lungs. The effect has been observed in various species with such compounds as DFP (Modell *et al.*, 1946; Heymans and Jacob, 1947), HETP (Dayrit, Manry, and Seevers, 1948), TPP (Verbeke, 1949), and tabun (Holmstedt, 1951), and is often of such severity as to be suggested as the cause of death (Koelle and Gilman, 1949). This lessened distensibility of the lungs is generally attributed to bronchoconstriction.

The occurrence of bronchoconstriction in anticholinesterase poisoning has been demonstrated radiographically. Rabbits were anaesthetized with urethane, 0.5 ml. of lipiodol introduced into the exposed trachea, and its spread watched on a fluorescent screen. With sarin, given intravenously, evidence of narrowing of the respiratory tubes was obtained at all dosage levels. With 100 μg./kg. sarin, narrowing of the bronchi and of all sizes of bronchioles occurred, and this was maximal 1 min. after the injection.

A quantitative evaluation of the degree of bronchoconstriction has also been attempted in rabbits. The animals were anaesthetized with urethane and were maintained on negative pressure ventilation by enclosing them in a Drinker-type machine which permitted a rhythmical reduction of pressure, constant in rate (52/min.) and extent, throughout the experiment. The chest was opened by splitting the sternum in the mid-line and the tidal air was measured by connecting the tracheal cannula to a rebreathing circuit comprising a soda-lime canister, gas circulator, and small recording spirometer. The artificial respiration was adjusted so that spontaneous respiratory movements continued.

After 40 μg./kg. sarin, intravenously, the pattern of response was similar in 12 out of 14 rabbits, the tidal air falling away within half a minute of the injection, reaching an average minimum value of 40% of normal at about 2 min., and thereafter gradually increasing, so that by the third minute the tidal air was 55% of normal. In the other two rabbits the tidal air was only transiently diminished and the minute volume was hardly affected. The reason for this different behaviour was not apparent; both these animals showed other characteristic signs of anticholinesterase poisoning, such as muscular twitching and the cessation of spontaneous respiratory movements.

Bronchoconstriction has been demonstrated in the decapitate cat and in the dog heart-lung preparation. In the latter, however, the addition of acetylcholine to the circulating blood may be required. The explanation of the bronchoconstriction in anticholinesterase poisoning has been disputed. It has been attributed to an action on the vagal ganglion cells (Verbeke, 1949; Heymans, 1949) or to a direct action on the effector cells (Koelle and Gilman, 1949) or to the accumulation of acetylcholine at the vagal postganglionic nerve endings (Koelle and Gilman, 1949).

By the use of twin preparations of guinea-pig trachea (permitting the necessary control observations) it has been shown that sarin, DFP, and TEPP, in high dilution, not only sensitize the isolated muscle to acetylcholine, but throw it into spasm just as eserine does. Their tracheal spasmogenic potency parallels their ability to inhibit cholinesterase (Koppanyi, Karizmar, and King, 1947), and the tracheal contractions induced show features—such as long latency and slow development—which have been held to characterize drugs acting indirectly by permitting the accumulation of acetylcholine (Adrian, Feldberg, and Kilby, 1947).

There is no doubt, therefore, that anticholinesterases can produce bronchoconstriction, but it is doubtful if this is the main, general cause of failure of ventilation. Furthermore, although failure of respiration is the cause of death in all species, the severity of the bronchoconstriction produced by sarin, tabun, E600, DFP, and TEPP seems to vary with the species, being, at the time of acute failure, slight in rabbits, severe in cats, and often insignificant in monkeys. Thus, although in rabbits the degree of bronchoconstriction pro-

duced by sarin reduces the tidal air to 40% of normal 2 min. after poisoning, the bronchoconstriction produced in cats (pentobarbitone anaesthesia) by tabun, sarin, E600, DFP, and TEPP is much more severe. Records of tidal air, intrapleural pressure, and phrenic nerve discharge show that immediately after injecting one of these drugs a marked degree of bronchoconstriction develops. The cat then makes more powerful inspiratory efforts and the tidal air increases again, but then respiration ceases abruptly (Fig. 1). With a large dose of anticholinesterase, bronchoconstriction may be complete, inspiratory efforts continuing without any resulting air intake.

In the monkey anaesthetized with pentobarbitone, the bronchoconstriction produced by the compounds listed develops more slowly than in the cat and does not become so severe. It seems evident, therefore, on the basis of the data presented, that the failure of ventilation after intoxication with an anticholinesterase cannot be attributed solely to loss of distensibility of the lungs from bronchoconstriction. Can it be explained by an insufficiency of the respiratory "bellows" mechanism?

Production of Neuromuscular Block.—The activity of the respiratory muscles during anticholinesterase poisoning has been studied in some detail in the rabbit anaesthetized with urethane. In one series of experiments the phrenic nerve was stimulated supramaximally at 50–70/sec. every other second and the diaphragm contractions were recorded by means of the phrenograph, the rabbit being artificially ventilated with the chest open. Fig. 2 shows that, within 5 seconds from the end of the intravenous injection of 40 μg./kg. sarin, the height of the tetanic response of the diaphragm to stimulation of the phrenic nerve was much reduced. Ten seconds after the injection the diaphragm had ceased to respond tetanically, giving only a single twitch to the first of each train of impulses. A well maintained tetanus could, however, still be obtained by direct stimulation of the diaphragm. As would be expected, the twitch response of the diaphragm to single shock stimulation of the phrenic nerve (one

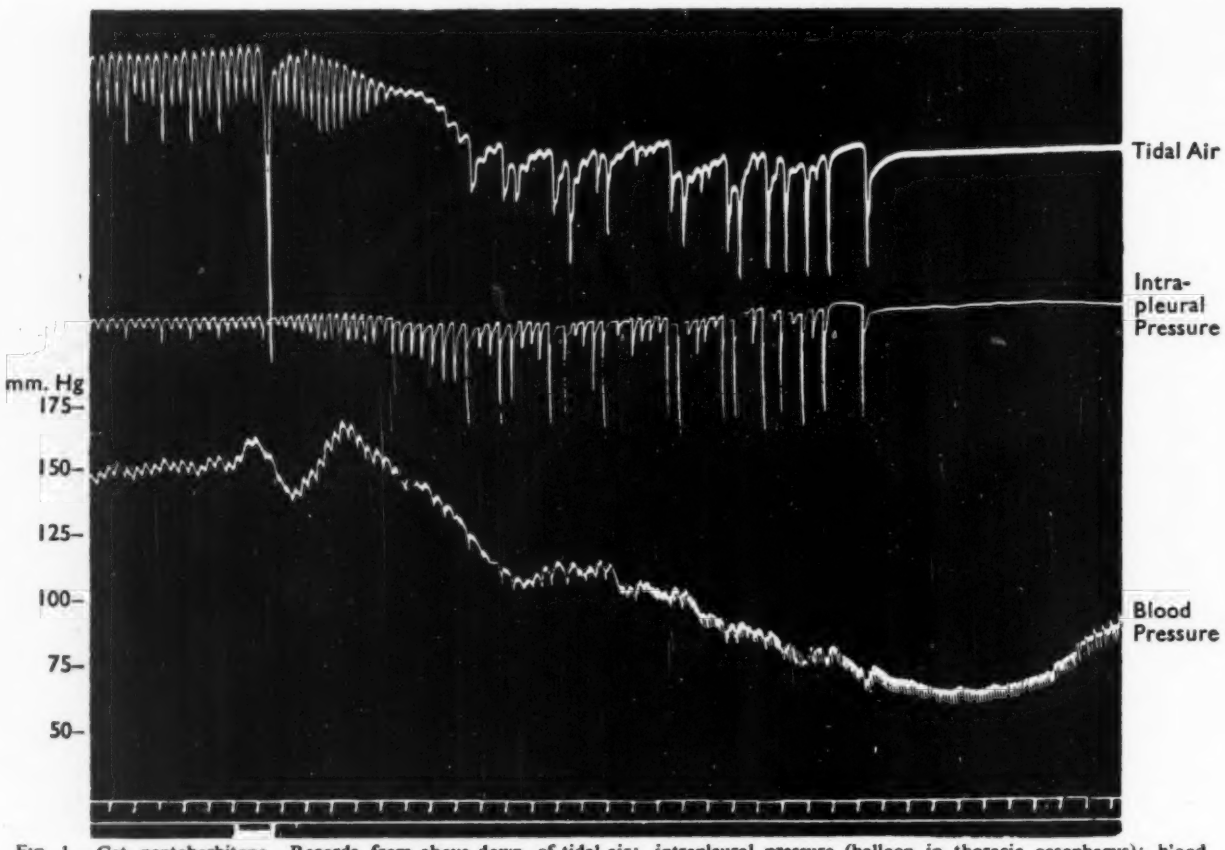
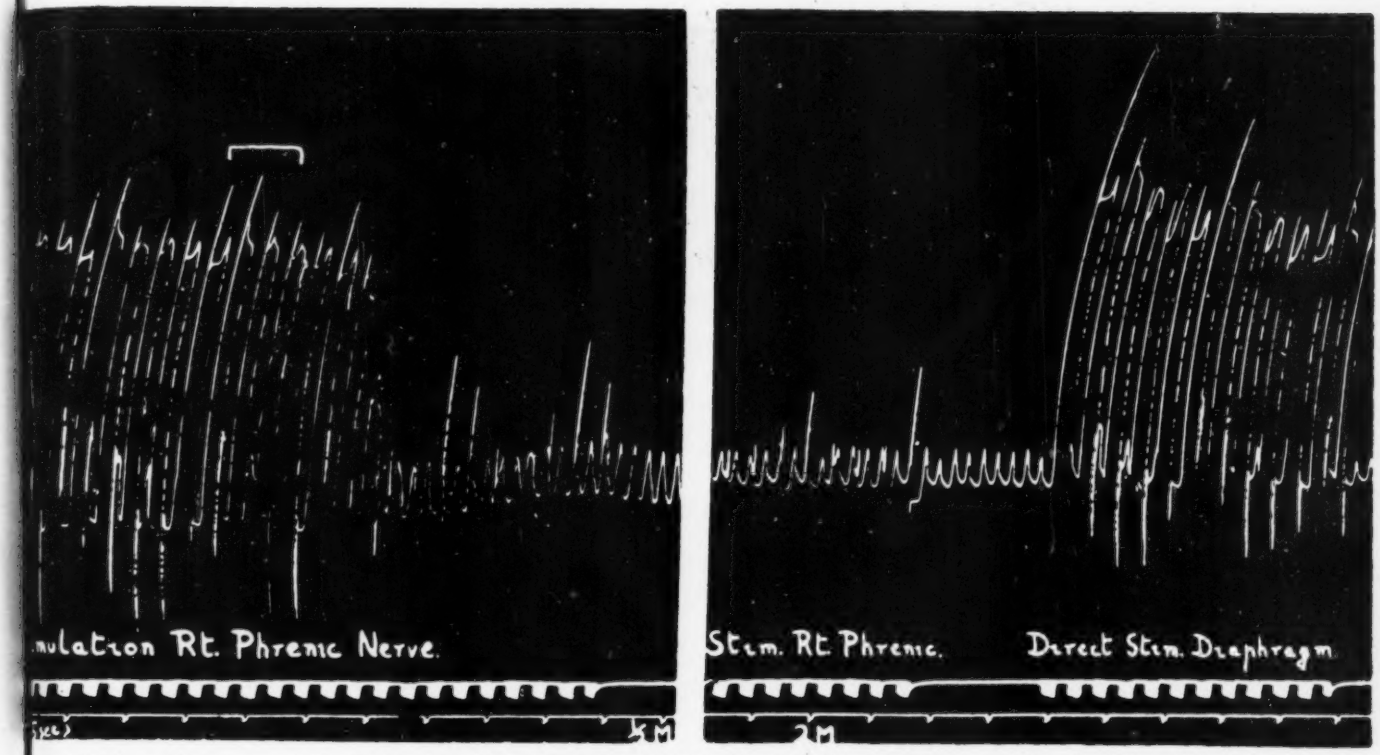


FIG. 1.—Cat, pentobarbitone. Records, from above down, of tidal air; intrapleural pressure (balloon in thoracic oesophagus); blood pressure (femoral artery). E600, 1.5 mg./kg. injected intravenously at signal. Time-marker, 5 sec.



2.—Rabbit, urethane, open chest. Contractions of diaphragm (recorded by phrenograph) in response to repetitive stimulation of right phrenic nerve. 40 μg./kg. sarin injected intravenously at upper signal. The effect of direct stimulation of the diaphragm 2 min. 20 sec. from the injection is shown on the right. (The small pointed waves were caused by displacement due to the artificial respiration.)

stimulus every 10 sec.) was not abolished by 40 μg./kg. sarin, being first increased and then only slightly reduced.

The block to rapidly repeated indirect stimulation (an example of Wedensky inhibition; Wedensky, 1885; Adrian, 1913) has also been found to occur in the isolated phrenic-diaphragm preparation of the rat (Bülbring, 1946) with eserine, neostigmine, DFP, TEPP, tabun, sarin, soman, E600, and other anticholinesterases (Evans, 1951). On washing, the inhibition from eserine and E600 was removed easily, from TEPP more slowly, but from DFP only when the concentration was low and the exposure time short.

Thus, undoubtedly, neuromuscular block can and does develop rapidly after sufficiently intense anticholinesterase poisoning, but the results from the isolated diaphragm, and from the stimulation experiments described above, cannot be taken to apply generally to the intact animal breathing spontaneously. Repetitive stimulation of the phrenic nerve for some considerable time before the injection of the drug would result in an abnormally high activity of the acetylcholine neuromuscular transmission mechanism and thus predispose it to block (see, for example, Burns and Paton, 1951). Such stimulation would also be expected to cause vasodilatation in the diaphragm, perhaps resulting in a greater amount of the drug reaching the muscle than would normally be the case. Moreover, if neuromuscular block were the sole cause of respiratory failure, it would be difficult to account for the action of atropine in preventing death from anticholinesterase poisoning, since atropine does not relieve the neuromuscular block caused by anticholinesterase drugs.

It is therefore necessary to study the results of experiments in which the physiological state of the animal was undisturbed until the immediate effects of the drug were over. The right phrenic nerve in the neck of a rabbit was placed on silver-silver chloride stimulating electrodes, *but was not cut.* Sarin, 40 μg./kg., was injected into the femoral vein, and, immediately after spontaneous respiration had ceased, the phrenic nerve was stimulated supramaximally at 30/sec. for periods of 1 second, the diaphragm response being recorded by the phrenograph. Control diaphragm contractions were obtained at least 5 min. before the injection by stimulating the nerve during the apnoea following forced hyperventilation. The stimulus rate was 30/sec., because this is about the

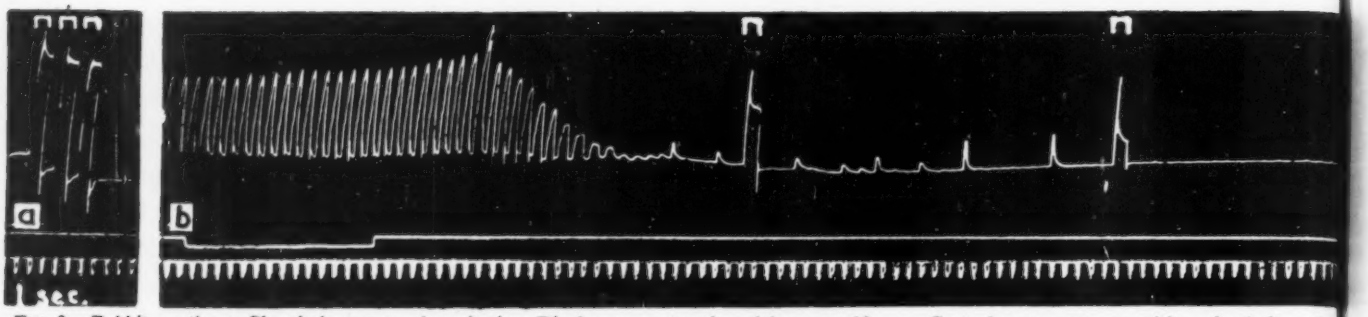


FIG. 3.—Rabbit, urethane. Closed chest, normal respiration. Diaphragm contractions (phrenograph). *a*.—Control response to repetitive stimulation of p phrenic nerve during apnoea after forced hyperventilation. *b*.—Spontaneous diaphragm contractions and responses to stimulation (upper signal) a sarin 40 μg./kg. intravenously at signal. Time, 1 sec.

normal rate of discharge of the spontaneous diaphragm action potentials of an anaesthetized rabbit breathing quietly. In a study of neuromuscular block this would be important, since any developing block at the neuromuscular junction will prevent the transmission of stimuli of higher frequencies while still allowing those of normal frequency to pass.

In rabbits given sarin 40 μg./kg. the development of neuromuscular block varied considerably without parallel variations in the picture of respiratory failure. In some experiments there was little, if any, block at the diaphragm after spontaneous contractions had ceased, whereas in others (Fig. 3) neuromuscular block developed more rapidly but was still not sufficient to account for the initial rapid reduction in diaphragmatic activity.

Production of Central Respiratory Failure.—Since neither neuromuscular block at the diaphragm nor bronchoconstriction appears to be the sole cause of respiratory failure in the rabbit, experiments were done in which the action potentials in the upper root of the right phrenic nerve and the diaphragm contractions of the opposite side were recorded. Figs. 4A and 4B are records of a typical experiment, and show that there is a rapid diminution of the impulse traffic in the phrenic nerve after the injection of sarin, 40 μg./kg., with no further discharge after the diaphragm contractions and resultant air intake have stopped. It would appear from this that the respiratory motor neurones have ceased firing and that the diaphragmatic paralysis results from this and not from a peripheral block.

This central action is also shown with the other anticholinesterases used, although there may be differences in the relative rates at which they stop the central discharge and produce block at the neuromuscular junction. Tabun, 0.15 mg./kg. i.v., resulted in complete neuromuscular block at the rabbit diaphragm 15 sec. after the injection. This led to an *increased* phrenic nerve discharge from central stimulation by asphyxia and by vagal or other afferent nervous discharge (Daly and Wright, 1953). The stimulation, however, was insufficient to overcome the gradual depression of phrenic nerve discharges from the direct effect of the drug on the central respiratory mechanism, which caused the phrenic nerve impulses to cease 25 sec. from the injection. DFP, 4 mg./kg. i.v., stopped the central discharge of a rabbit 14 sec. from the start of the injection, thus resembling sarin (40 μg./kg.), whereas E600 (1.5 mg./kg.) and TEPP (300 μg./kg.) resembled tabun in that neuromuscular block developed rapidly before central failure had occurred.

Apart from any possible variations due to different rates of penetration of the various drugs, the relative rates of development of central inhibition and of neuromuscular block depend on the dose given. Daly and Wright (1953) have shown that with 30 μg./kg. sarin the discharge in a single

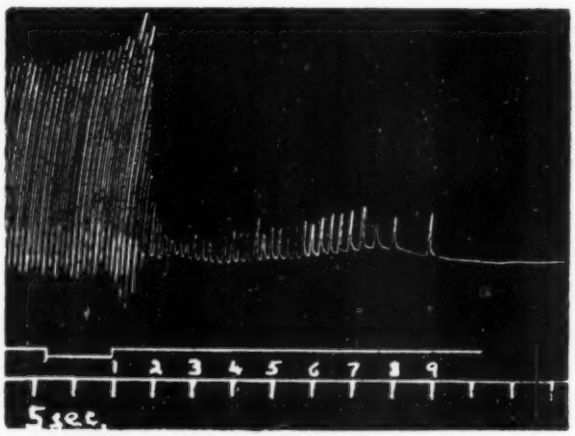


FIG. 4A.—Rabbit, urethane. Closed chest, normal respiration. Diaphragm contractions (phrenograph). Sarin, 40 μg./kg., injected intravenously at signal.

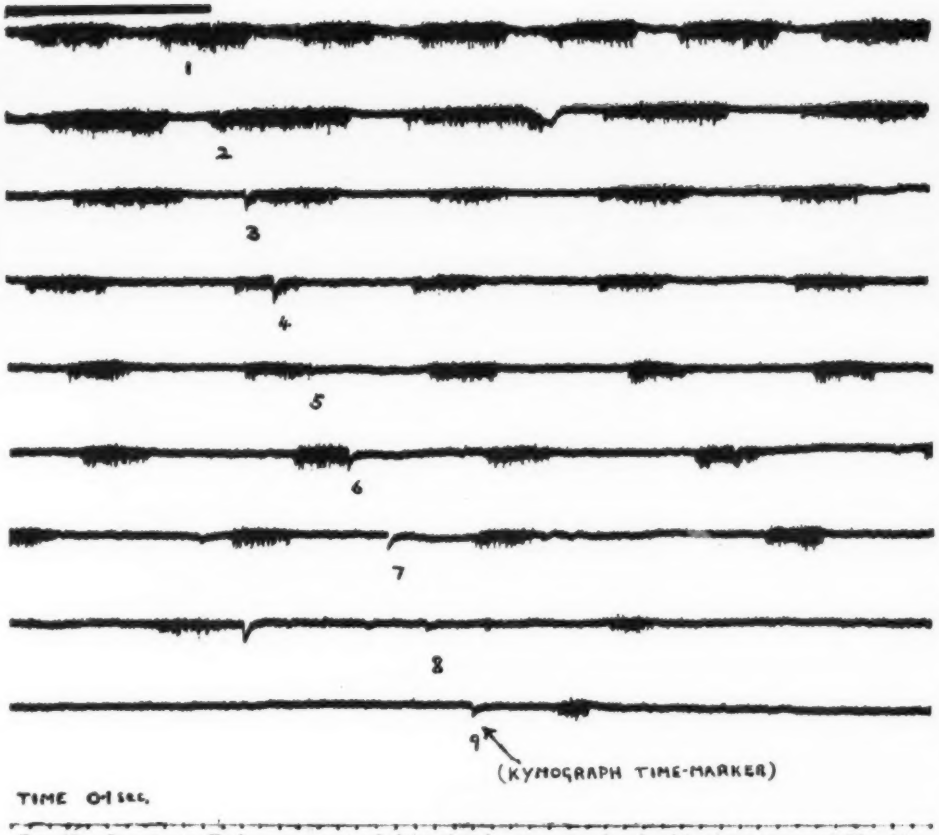


FIG. 4B.—Impulse traffic in upper root of right phrenic nerve associated with phrenogram of Fig. 4A. Numbers correspond to kymograph time-marker in Fig. 4A.

phrenic nerve fibre of a rabbit first increases before it finally fails. This has been confirmed, but with 40 μg./kg. the discharge has been found to fall off immediately without any initial increase. Both effects are shown in Fig. 5.

Species Variations in Response.—From the effects of anticholinesterase drugs on the respiration of rabbits described above, it can be concluded that respiratory failure is due primarily to an action of the drugs on the central respiratory mechanism, often associated with a considerable degree of neuromuscular block at the diaphragm. The chest muscles are less affected, so that chest movements of some magnitude may appear before and until the central inhibition is complete. Bronchoconstriction is variable but not usually severe in rabbits or monkeys.

As indicated above, a similar picture is not seen in other species. The most immediate effect of anticholinesterase poisoning in the cat is a marked bronchoconstriction. The consequent anoxia and hypercapnia cause a stimulation of the respiratory centre, and inspiratory efforts continue until the central discharge ceases abruptly.

According to Douglas and Matthews (1952), the factors responsible for respiratory failure in the cat poisoned with TEPP are paralysis of the respiratory muscles and failure of the respiratory centre, bronchoconstriction playing a secondary role. However, experiments with sarin, tabun, E600, DFP, as well as with TEPP, show that the neuromuscular block is only of secondary importance. Thus (Fig. 1) the large intrapleural pressures which develop after poisoning would not be possible if the respiratory muscles were inactive as a result of neuromuscular block. It was found

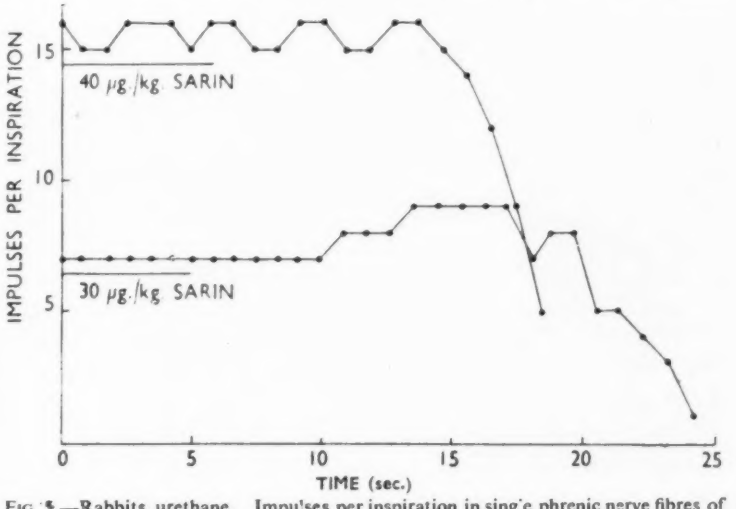


FIG. 5.—Rabbits, urethane. Impulses per inspiration in single phrenic nerve fibres of two rabbits, one given 30 and the other 40 μg./kg. sarin intravenously. The black line under each curve indicates the injection.

in some experiments that neuromuscular block did become complete at the diaphragm, but the picture of respiratory failure was nevertheless not changed, the important factor being that the chest muscles remained active. To demonstrate this, three experiments have been done with TEPP (0.3 mg./kg.) and two with E600 (1.5 mg./kg.) in which both phrenic nerves were cut before poisoning. The diaphragm was thus completely flaccid, but, until after the injection of the drug, adequate ventilation was maintained by the chest muscles alone. All five experiments showed the same result, of which Fig. 6 is an example.

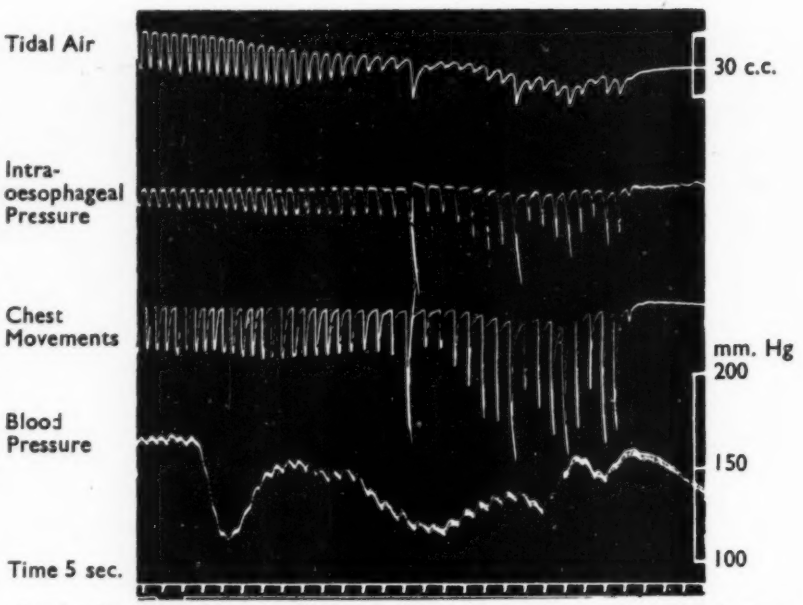


FIG. 6.—Cat, pentobarbitone. Closed chest, spontaneous respiration. Both phrenic nerves cut. Records from above downwards: tidal air, intrapleural pressure (intra-oesophageal balloon), chest movements (stethograph) (inspiration downwards for all three), blood pressure. Time, 5 sec. TEPP 300 μg./kg. intravenously at signal.

Even in this experiment where the chest muscles are more active than normally, there is evidently no significant degree of neuromuscular block; the immediate effect of the TEPP is to cause a marked bronchoconstriction, as shown by the increased intrapleural pressures with reduced air intake.

In monkeys respiratory failure in acute anticholinesterase poisoning, with all drugs used, appears to result entirely from inhibition of the central respiratory mechanism. The failure of respiration is characterized by a gradual reduction in the depth of inspiration, followed by an expiratory tone and forced expirations which pass off into apnoea (Fig. 7).

With lower doses of the drugs, the central inhibition is overcome by asphyxial stimuli, and, after periods of apnoea of up to 3¼ min., the apnoea is broken by inspiratory gasps. With sarin the inspiratory rate and depth gradually return to normal, but with E600 and TEPP, although the initial failure of respiration is the same as with sarin, the succeeding gasps are not so deep, and a record of diaphragm action potentials clearly shows some Wedensky block. The gasps were possibly not maintained with these two drugs because the air intake was insufficient to enable the severely strained heart to recover from anoxia.

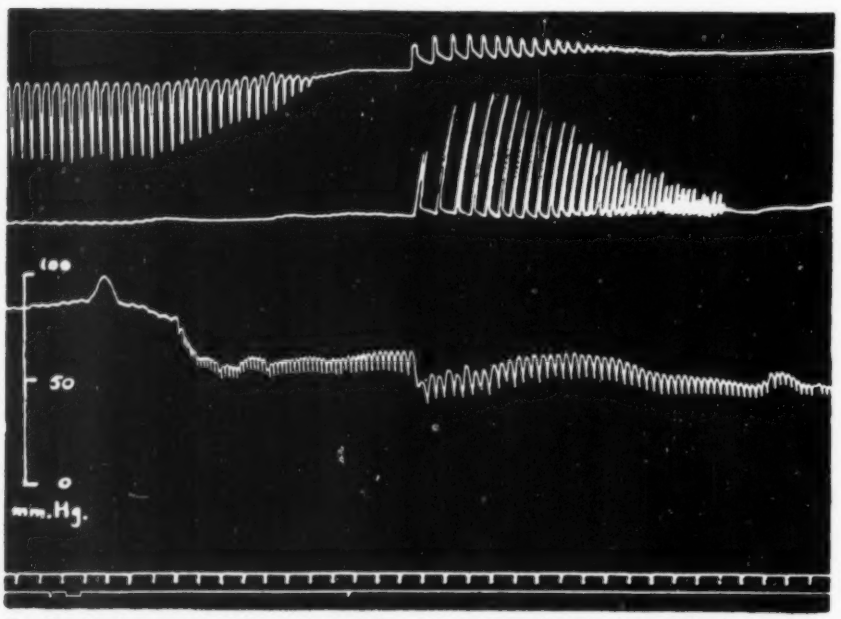


FIG. 7.—Monkey, pentobarbitone. Records from above down: tidal air; intra-abdominal pressure (inspiration downwards in both); blood pressure. Time, 5 sec. TEPP 250 μg./kg. intravenously at signal.

That the initial inhibition of inspiration results only from a central action of the

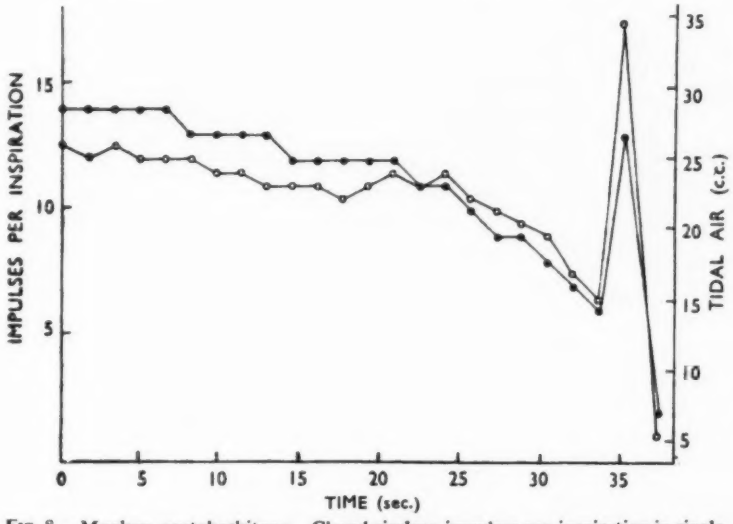


FIG. 8.—Monkey, pentobarbitone. Closed circles: impulses per inspiration in single phrenic nerve fibre (left-hand ordinate). Open circles: associated tidal air (right-hand ordinate). The curves start at the end of the injection of sarin 30 μg./kg. intravenously.

anticholinesterase is shown by the parallel reduction in impulse traffic in the phrenic nerve. If the reduction in air intake were due to bronchoconstriction or neuromuscular block, or both, the phrenic discharge would increase while the tidal air decreased. Fig. 8 shows that the number of impulses per inspiration in a single fibre of the phrenic nerve parallels very closely the air intake for each inspiration in the course of respiratory failure from sarin (30 μg./kg., i.v.).

The action of the anticholinesterase drugs in incapacitating the central respiratory mechanism is probably not due to reflex influences from the periphery, since injection of the drugs into the cisterna magna or into the vertebral artery of the dog also causes respiratory inhibition although the doses were so small (5 μg.) that no peripheral effects occurred. Nor does the respiratory inhibition appear to be due to repetitive excitation of the central vagal neurones (cf. Dirken and Woldring, 1951), since no increase in discharge from the central end of the vagus nerve was found at the time of failure of the phrenic nerve discharge. There were, however, abundant discharges down the vagus after inspiration had stopped, and in the monkey these coincided with the forced expirations (Fig. 7). If the inspiratory centre were inhibited reciprocally by activation of the expiratory centre, the respiration would be expected to change gradually to the forced expiratory type. Some of the kymograph and electrical records suggest, however, that the expiratory activity occurs *after* the inhibition of inspiration, and is therefore the result, not the cause, of the inhibition.

The respiratory inhibition could be explained as being due to direct inhibition of the phrenic motor neurones; but, if this were so, some large-scale inhibitory mechanism must be assumed, since the phrenic discharges, the inspiratory intercostal activity, and the "inspiratory" impulses in the recurrent laryngeal branch of the vagus all run and cease in parallel. The alternative, and more probable, explanation is that the "respiratory centre" itself is inhibited either directly or as a result of inhibitory impulses from some other part of the central nervous system.

Other Possible Factors Affecting Respiration.—There is another possible mechanism of respiratory failure which must be considered. The anticholinesterases may cause a profound fall in blood pressure, and it is conceivable that, with large doses, the blood pressure may be lowered to such a degree that the respiratory centre would fail because its blood supply proved inadequate. Thus, using cats anaesthetized with pentobarbitone, it has been shown repeatedly that 200 μg./kg. sarin intravenously will cause a rapid depression of the blood pressure to about 30 mm. Hg, but that the heart continues to beat, and to maintain circulation, for several minutes after respiration has stopped. On only one occasion was the heart stopped by sarin, and this was only transitory. Then a picture reminiscent of vagal inhibition and escape was seen. In spinal cats it was found that when the animal was maintained on a respiration pump the heart continued to beat after doses of sarin of 2,000 μg./kg.

However, the circulation might be slowed to such an extent that the respiratory centre failed owing to anoxia. The conditions of such anoxic failure of breathing were studied. The circulation in a cat was stopped completely by tightening a loose ligature which had been placed around the pulmonary artery. The last respiration occurred 150 sec. after tightening the ligature, which is about equal to the longest time ever seen for continuation of respiratory efforts following these large doses of sarin.

In the poisoned animals the blood pressure is depressed, but not completely abolished as it is in the control experiment. Therefore, in another experiment, the blood pressure of a cat was lowered by haemorrhage to 30–40 mm. Hg on two occasions, the first time for 180 sec. and, a

few minutes later, for 270 sec., the pressure being restored at the end of each period by re-injecting the blood into the circulation. Respiration continued throughout these periods. Thus it is clear that failure of the circulation is not adequate to explain the failure of respiratory effort. A further indication of this is the fact that, although respiratory arrest occurs in the rat poisoned with an anticholinesterase, the drug causes a large rise in blood pressure in this species.

One of the most striking effects of the anticholinesterase drugs in the conscious animal is the violent convulsions which occur. It was shown by Wescoe, Green, Macnamara, and Krop (1948) that DFP produces convulsive EEG patterns in curarized cats and monkeys, and it might be supposed that such a result might affect the picture of respiratory movements. During anticholinesterase poisoning from intravenous injection the respiration of conscious monkeys has been recorded using a face mask made air-tight with petroleum jelly and connected through valves to a spirometer. Respiratory failure occurred in the same general way as described above for the anaesthetized monkeys, though the picture was complicated by the addition of the convulsive movements.

Another central effect of the anticholinesterase drugs is an action on spinal cord reflexes (for review see Gerard, 1950). Chennells, Floyd, and Wright (1949), using myographic methods, found that TEPP facilitates various reflexes (knee jerk, flexor, peroneal and crossed extensor) and induces convulsions. The effects were due to a direct action of the drug on the central nervous system. The intravenous injection of a large dose of sarin (200 μg./kg.) to cats abolishes, within about 20 sec., the monosynaptic spike of the ventral root response elicited by maximal stimulation of the dorsal root. This is followed by inhibition of the polysynaptic spikes.

Holmstedt and Skoglund (1953) have obtained a similar inhibition of the monosynaptic spike following the intra-arterial injection of small doses of tabun, but find that there is an associated increase in the polysynaptic spikes. With intra-arterial DFP, both the monosynaptic and polysynaptic spikes were facilitated.

Summary

1. The effect of various anticholinesterase drugs in producing respiratory failure in mammals has been analysed.

2. All the drugs studied can impair ventilation in three main ways: by producing (1) bronchoconstriction, (2) neuromuscular block, and (3) central respiratory failure.

3. The central failure seems to be the predominant factor in most instances, but the detailed picture varies with the species studied, the drug used, and the dosage administered. Thus, in the rabbit, bronchoconstriction is slight and develops slowly, while neuromuscular block may be severe at the diaphragm but is less marked at the chest muscles. In the cat, bronchoconstriction may be early and severe, and again, although neuromuscular block may occur at the diaphragm, the chest muscles retain their activity until central failure occurs. In the monkey, central failure appears to be the sole cause of cessation of ventilation, the bronchoconstriction and neuromuscular block being insignificant at the time of respiratory failure.

4. The depression of the activity of the respiratory centre is apparently due to an action of the anticholinesterase within the central nervous system.

5. Atropine will protect against the central inhibition and the bronchoconstriction, and artificial ventilation will maintain life in the presence of neuromuscular block.

Acknowledgment is made to the Chief Scientist, Ministry of Supply, for permission to publish this work.

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DEXTRAN SULPHATE: THE RELATION OF MOLECULAR FEATURES TO BIOLOGICAL PROPERTIES

BY

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(RECEIVED AUGUST 25, 1953)

In a previous paper (Ricketts, 1952a) a series of dextran sulphate preparations varying widely in molecular weight and sulphur content was described. The biological properties of these compounds were examined by Walton (1951, 1952), who found that molecular weight was the principal feature determining toxicity, the smaller molecules being the least toxic. At molecular weights (M_N) of the order of 7,000 a blood anticoagulant activity of about 15 international heparin units per mg. was attained when the degree of sulphation exceeded an average of 1.3 sulphate groups per glucose unit. It is in this region, where therapeutically useful anticoagulant activity is to be found, that a more detailed exploration of the relation between molecular features and biological properties than was provided by earlier work is necessary. The fractional precipitation of dextran sulphate from solution brings about, to some extent, a separation of molecular sizes. Exploration of the biological properties of such fractions provides an indication of the optimum molecular weight distribution in dextran sulphate intended for clinical use as an anticoagulant.

MATERIALS AND METHODS

Sulphur content was determined as previously described (Ricketts, 1952a). Intrinsic viscosity was measured in a No. 1 B.S.S. viscometer at 37° C. Over the range of concentration 1 to 4% in a 0.9% sodium chloride solution the plot η sp.*/c against c was linear, the intercept being the intrinsic viscosity.

Anticoagulant activity was measured as follows: sodium dextran sulphate 60 to 120 μg. in 0.1 ml. saline mixed with oxalated horse plasma (1 ml. 0.1M-potassium oxalate per 9 ml. blood) was incubated for 30 minutes at 37° C. Thrombokinase solution, 0.1 ml. (Difco, diluted 1:10 to 1:400 as required to give convenient clotting times), was added, followed by 0.1 ml. 0.025M-calcium chloride solution. Tubes were tilted at minute intervals and clotting was recorded up to 20 minutes. If clotting occurred at the same time in two tubes they were taken to contain the same number of units of anticoagulant activity. Intermediate values were obtained by interpolation. Accuracy is limited, because the observations are at minute intervals, to about ±15%.

Samples of dextran sulphate powder contained 8 to 15% moisture, which was determined for each sample by drying to constant weight *in vacuo* over phosphorus pentoxide at 60° C. The figures of Table I are corrected for this moisture content.

The precipitation of fibrinogen and other proteins from blood plasma is a limiting factor in the use of sulphated polysaccharides as clinical anticoagulants (Walton, 1952). This had previously been found to occur with dextran sulphate having a molecular weight in excess of about 20,000. Its occurrence in occasional samples of lower molecular weight suggested that such samples were unusually polydisperse. This possibility was investigated by fractional precipitation of one such sample with ethanol.

The fractionation was carried out as follows: ethanol was added to 15.4 g. of sodium dextran sulphate in 77 ml. 0.72% sodium chloride buffered with sodium bicarbonate at *p*H 7.3, to incipient turbidity at 30° C.; 108 ml. was required. The mixture was warmed until it became homogeneous at 33° C., and allowed to cool slowly. The precipitated syrup was separated and triturated in ethanol to a powder, fraction 1. Successive fractions were obtained similarly after the addition of further amounts, 2.5 to 25 ml., of ethanol.

RESULTS

Table I summarizes the properties of the initial sodium dextran sulphate and the seven fractions derived from it. Consideration of this table shows that 77% of the initial material is accounted for in these fractions. There was reason to suppose that the larger molecules would appear in the earlier fractions, and the intrinsic viscosity measurements show that this occurred. If, as seems likely, intrinsic viscosity is an additive property in this system, nearly the whole (80.2%) of the intrinsic viscosity can be accounted for. Material remaining in the solution at the final ethanol concentration, 52% v/v, was most probably of very short chain length and correspondingly low intrinsic viscosity. There is remarkably little variation in the sulphur content of the fractions until fraction 7, which has appreciably less

*η sp. is specific viscosity.

TABLE I
FRACTIONATION OF DEXTRAN SULPHATE

Fraction No.	% by Weight	Intrinsic Viscosity	%S in Na Salt	Activity (Units per mg.)	Fibrinogen Precipitation	Deposit in RE Cells
Original material	—	0·044	17·9	16·5	+	+
1	14·69	0·070	19·4	20·7	+++	+
2	11·44	0·058	19·3	20·2	++	+
3	10·25	0·054	20·6	20·7	++	+
4	11·23	0·044	19·4	17·7	+	+
5	15·44	0·035	19·6	14·2	0	0
6	9·83	0·026	19·3	11·9	0	0
7	3·95	—	14·9	4·6	0	0

sulphur. In all, 82.9% of the original sulphur is accounted for.

Comparisons of the original dextran sulphate with the International Standard Heparin preparation have indicated an activity close to 15 u./mg., or 16.5 u./mg. dry basis. This figure was assumed for the purpose of comparing the fractions with the original material. The sum of the activities of the fractions accounts for 80% of the original activity. From these measurements anticoagulant activity is seen to decrease slightly with decreasing intrinsic viscosity in the range 0.054 to 0.026.

The occurrence or otherwise of precipitation of fibrinogen *in vitro* by these fractions was investigated under physiological conditions of temperature, *p*H, and ionic strength by the technique previously described (Walton, 1952). Plus signs in Table I denote the extent of the precipitation observed.

It has previously been shown that similar precipitation occurs *in vivo*. The insoluble dextran sulphate protein complex formed in the blood stream is removed by the phagocytic activity of reticulo-endothelial cells in the liver, spleen, and bone marrow, and may be detected histologically in sections stained with toluidine blue (Walton, 1951).

It will be seen from the table that fibrinogen precipitation persists down to fraction 4 and that in this respect there was satisfactory correlation of the *in vitro* and *in vivo* tests for its occurrence.

DISCUSSION

Considering firstly the factors which affect fractionation, the solubility of a molecular species of dextran sulphate in ethanol-water solution is determined by the concentration of each component, temperature, ionic strength, and *p*H. Theoretically, dextran sulphate molecules may vary in length, and possibly in degree of branching, as well as in average degree of sulphation and uniformity of distribution of sulphate groups. Some limitation is, however, imposed by the relatively small number of glucose units in each molecule and by the comparatively high degree of sulphation. Provided sufficient electrolyte be present to avoid anomalies in viscosity, and to give a linear relation of η sp./c with c, the intrinsic viscosity so obtained is probably a measure of the length of the molecule. Although slightly lower intrinsic viscosities may be obtained in molar sodium chloride, there is probably some value in figures obtained in 0.9% sodium chloride, since activity measurements and other biological tests are made close to this ionic strength. Use of the original material as a standard of comparison for anticoagulant activity helps to overcome uncertainties about the validity of comparing different substances in a biological assay. Although the accuracy of the activity assays is not high, the progressive fall of anticoagulant activity with intrinsic viscosity is undoubtedly real.

For a clinical anticoagulant, the highest activity consistent with the absence of fibrinogen precipitation and its associated pathological effects is required. The indications are that this would be provided by preparations similar in molecular composition to fractions 5 and 6. When these fractions were injected into rabbits the expected elevation of blood clotting time occurred. In view of the results with sulphuric esters of oligosaccharides in the maltose series, which displayed anticoagulant activity *in vitro* but were relatively ineffective *in vivo* (Ricketts, 1952b), it would be of interest to explore the properties of even smaller dextran sulphate molecules, and this is being done.

SUMMARY

1. Earlier work indicated that the smaller molecules of dextran sulphate might be useful anticoagulants. This paper describes more precisely the gradation of biological properties with molecular size.

2. Sodium dextran sulphate having about two sulphate groups per glucose unit was subjected to fractional precipitation from ethanol-water solution. A fraction containing 19.6% S and having an intrinsic viscosity of 0.035 showed an anticoagulant activity of 14.2 units per mg., and was free from undesirable properties.

Thanks are due to Mr. M. Hall for his help in the laboratory. We are grateful to Messrs. Dextran Ltd. for the gift of a specially graded preparation of dextran from which the dextran sulphate used for these experiments was readily prepared.

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Printed in Great Britain by Fisher, Knight & Co., Ltd., The Gainsborough Press, St. Albans.